Physiological effects of copper, cadmium and reduced salinity on intertidal and cultivated *Perna canaliculus* mussels

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Frantz E. Smith

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Abstract

The endemic mussel *Perna canaliculus* is the most valuable aquaculture product in New Zealand. Limited information has been compiled on physiological functions such as clearance, respiration and excretion of this species. Scope for Growth (SfG) which is the difference between the energy acquired from feeding and the expenditure of energy on respiration and excretion has been determined for this species from various locations in New Zealand. Existing information suggests that SfG shows limited correlation with the distribution, origin or contamination of mussels in their habitats. Therefore, the aim of the thesis research was to investigate the SfG of farm and intertidal mussels and compare how these values responded to challenges from reduced salinity and exposure to the trace metals copper and cadmium.

Preliminary experiments on mussel respiration rates (ml O_2 g^{-1} h^{-1}) showed that recently collected mussels respired at a rate of 0.222 $\pm$ 0.013 ml O_2 g^{-1} h^{-1} while mussels held for two weeks under laboratory conditions respired at 0.098 $\pm$ 0.009 ml O_2 g^{-1} h^{-1}. Respiration rates of recently collected farm and intertidal mussels measured every four hours over 28 hours were also found to be similar. The exponent (b) in the allometric relationship between respiration and dry weight was 0.5766. Respiration rate and condition of mussels which were maintained with feeding for two month was greater than respiration for mussels which were not fed over this time period. Specific dynamic action (SDA) was found to be the difference between the pre-feeding respiration rate of 0.15 $\pm$ 0.02 ml O_2 g^{-1} h^{-1} and 1.03 $\pm$ 0.16 ml O_2 g^{-1} h^{-1} at one hour after feeding. Respiration returned to pre-feeding rates between two and four hours after feeding was stopped.

Mussels exposed to 0, 1, 10 and 100 μg Cu L^{-1} showed no physiological effects of copper. Exposure of intertidal mussels to 1,000 μg Cu L^{-1} caused 100% mortality after three days and exposure to 500 μg Cu L^{-1} caused 100% mortality after seven days. There was 100% mortality of farm mussels exposed to either 500 μg Cu L^{-1} or 1,000 μg Cu L^{-1} after three days. The time for 50% mortality (LT_{50}) of intertidal mussels exposed to 500 μg Cu L^{-1} was 3.4 days. For an exposure of 250 μg Cu L^{-1}, the LT_{50} was 5.2 days.

SfG of intertidal mussels exposed to 100 μg Cu L^{-1} decreased by 91% for intertidal mussels in full salinity but SfG of farm or intertidal mussels exposed to cadmium at concentration of 33 μg Cd L^{-1}, 66 μg Cd L^{-1} or 99 μg Cd L^{-1} was not affected. Significant effects of cadmium were detected at a concentration of 1,000 μg Cd L^{-1} which resulted in a reduction in SfG by 60% compared to the controls. The initial experiments showed that copper resulted in lower SfG than cadmium at similar concentrations.

For mussels exposed to combinations of 0 and 100 μg Cu L^{-1} at 34 ppt and reduced salinity (17 ppt) the SfG of intertidal specimens exposed to 100 μg Cu L^{-1} at 34 ppt was decreased by 85%. SfG of farm mussels exposed to this level of copper was reduced by 70%. The effect of copper on intertidal mussels may be related to the higher clearance rate of these mussels. In the experiments with cadmium, exposure to 1,500 μg Cd L^{-1} in full salinity resulted in a decline in SfG in excess of 100% for farm mussels but only 91% for intertidal mussels in full salinity.

The effect of reduced salinity as the only stressor was also noted in these experiments. In both the copper and cadmium experiments, the treatments exposing mussels to 50% salinity resulted in severe declines in SfG for both farm and intertidal mussels. The decline was 99-100% for farm mussels and 60-82% for intertidal over fourteen days. In addition to salinity, the effect exposure time significantly affected the physiology of intertidal mussels in low salinity.

In treatments which combined 50% salinity with either 100 μg Cu L^{-1} or 1,500 μg Cd L^{-1}, SfG of farm mussels declined into negative values. SfG of intertidal mussels exposed to 100 μg Cu L^{-1} or 1,500 μg Cd L^{-1} in low salinity declined by 87% and 98% respectively. The combination of salinity and metals resulted in significant interactive effects but these were not synergistic effects. This suggested that salinity could be a major source of variability in SfG of mussels which are exposed to similar concentrations of metals. The experiments also showed that intertidal mussels possessed greater physiological adaptability to low salinity. This suggests that responses to salinity differ from responses to trace metals. Salinity is therefore an important modulator of the sub-lethal effects of toxic trace metals such as copper and cadmium.

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Chapter 1 General Introduction

1.1 Importance of *Perna canaliculus*

There are sixteen species of native and introduced mussels recorded in New Zealand (Seager, 2005). These occupy natural substrates such as rocks and reefs (Alfaro, 2001) as well as man made structures such as wharf piles, mooring buoys, and pipes along the coast (Hickman, 1979). Among these sixteen species, the endemic *Perna canaliculus* grows best and is dominant in subtidal habitats (Knox, 1953; Hickman, 1979). Contiguous beds which develop from the subtidal niche dominated by *P. canaliculus* can extend to cover swaths of hundreds of meters of coastline. These mussel beds provide habitats for rich communities of small invertebrates as well as birds and predatory vertebrates which also occupy New Zealand's coasts.

Perna canaliculus is known to be a proficient consumer of primary productivity and can cause detectable depletions of phytoplankton biomass where high concentrations of the mussels occur (Ogilvie *et al.*, 2000). This high level of feeding plays an important role in the transfer of marine primary productivity to higher levels in the food chain (Gibbs, 2004). The high productivity of *P. canaliculus* is an important reason why this species has become the species of choice for the NZ $150 million mussel farming industry in New Zealand. However, continuous access to natural productivity in the low intertidal niche also means that *P. canaliculus* is the mussel species with the greatest chance of contact

with waters which may become contaminated by chemicals or organic effluents arising from human activities.

1.2 Commercial importance

Perna canaliculus was used by Maori as easily accessed protein (Teviotdale, 1931; Hauraki Maori Trust Board, 1999) and the species has been commercially harvested since the late 1800s. Overexploitation of the resource eventually lead to decline and closures of dredge fisheries in the 1960's (Dawber, 2003). In the 1970's, marine farmers began to experiment with transferring spat from the North Island to cultivation sites in Nelson, Firth of Thames and Stewart Island (Dawber, 2003). Based on the success of this model, *P. canaliculus* has become the most commercially important of the cultured molluscs in New Zealand. The product is marketed as New Zealand Greenshell^{R} mussels and is New Zealand's flagship aquaculture product in terms of export earnings. Marine farmers have built a reputation of being able to supply a product of high quality to United States markets which purchase 70% of the product. The industry reliably earns over NZ $150 million annually and typically provides 2,330 full time equivalent employment in Marlborough alone (Anon., 2000). Upstream linkages to the economy include support for dockyards, ice suppliers, specialized marine engineering service providers, packaging suppliers, research agencies, universities, quality assurance services, plastics and rope manufacturers. Linkages downstream include supply of inputs to processing plants, marketing and brokerage providers, shipping lines, and the neutraceutical manufacturing sector which has developed a number of value added products marketed for arthritis (Gibson and Gibson, 1998) and skin health.

Export earnings from *Perna canaliculus* have steadily grown from the modest levels of the 1970s when an export market was being developed (Dawber, 2003). By 1986, the industry earned NZ $12 million (Waite, 1989). Recently, production has increased beyond the 30 tonnes produced in 2004 (Table 1.1).

Table 1.1: Quantity (tonnes) and value $ NZ of *Perna canaliculus* mussels from New Zealand.

Year	Quantity (Tonnes)	Value (NZ$ millions)
2000	28,100.00	169.2
2001	20,900.00	157.5
2002	28,800.00	185.3
2003	27,900.00	133.2
2004	30,277.00	141.4
2005	34,008.60	166.6
2006	35,143.00	182
2007	36,114.50	174.5

Earnings in 2000 were NZ $169 million but this declined to NZ $133 million in 2003. Income rebounded in 2005 and has remained over NZ $150 million since. Stable export earnings in the face of a slight decline in production is attributed to strong demand for New Zealand product. The 2002-2004 two year price for New Zealand Greenshell^{R} medium size product was US $3.88/kg but the 2005 price for the same product on April 15, 2005 was US $3.972/kg (www.comtell.com, 2005).

In addition to aquaculture, *P. canaliculus* mussels have been used by Maori to make ornaments and fishing implements. In more recent times, *Perna canaliculus* mussels have also been used to manufacture neutraceutical tablets rich in polyunsaturated fatty acids (Taylor and Savage, 2006). *P. canaliculus* has also been used by commercial resource consultants to assess the environmental impacts of roads in New Zealand (Kennedy, 2003). In one study, *P. canaliculus* mussels from Wellington City were found to contain elevated levels of lead compared to samples from Taranaki. The tissue of *P. canaliculus* has also been used to quantify persistent organic pollutants (POPs) and trace metals in New Zealand (Kennedy, 1986; Rainbow, 1995).

1.3 Threats to the industry

The major threat to the New Zealand aquaculture sector is that the high value of the floating exchange rate makes the price of New Zealand Greenshell^{R} mussels unfavorable to US buyers compared to product from Chile or Spain. The main biological problem facing the New Zealand mussel industry involves the occurrence of harmful algal blooms. During these blooms, a moratorium is placed on harvesting and sale of mussel spat as well as mussels for human consumption.

Because of proper planning and good water quality, there have been few serious threats to the aquaculture industry in New Zealand. However, a number of conflicts based on use of space to harvest or culture the product have arisen (Bess, 2006). These conflicts include legal action brought by the Waikare Inlet oyster farmers against the Far North District Council whose Kawakawa sewage plant was said to be responsible for viral contamination of oysters in 1994, 1999, and 2001. Sewage spills have occurred as recently as 2007.

These occurrences suggests that the availability of accessible space to produce *Perna canaliculus* in New Zealand may be diminishing (Gibbs, 2004). Such a pattern has occurred in North America and Europe where space available for aquaculture has declined and where water quality has also declined (Gibbs, 2004). For example, the bivalve aquaculture sector in the Hong Kong (Goldberg, 1990), France (Alzieu, 2000), Taiwan (Chao *et al.*, 2005), Thailand (Elfwing and Tedengren, 2002a) and Canada (Kruzynski, 2000) have all had notable problems with declining water quality when these industries grew. These problems have included elevated nitrogen and phosphate levels in estuaries impacted by agricultural watersheds (Wade *et al.*, 2004). In addition to effects on estuaries, pollution resulting from large scale alterations of land or industrial dumping has lead to elevated levels of pollutants at offshore locations on the continental shelf (Mil-Homens *et al.*, 2006) which receive runoff even from small rivers (Gaston *et al.*, 2006). Such pollution of marine areas under terrigenous influence is said to be widespread and an important global challenge of the coming years (Nellemann *et al.*, 2008). Pollution is believed to be a significant factor behind the disappearance of mussels from the pleasure boat harbors of Vieux-Port and Pointe-Rouge in Marseille and Cannes (Guillon-Cottard *et al.*, 1998). Mussels have also disappeared from Barcelona Harbour, and mass mortalities of cultured *Ostrea edulis* in Catalan waters in 1997 caused a shift from culturing this species to *Crassostrea gigas* (Ramón *et al.*, 2005).

1.4 Trace metals: a global concern

The concerns addressed in this thesis arose from the recognition of a global trend of increased marine pollution in the coastal zones of the world where most of the important fishing and aquaculture grounds are located (Nellemann *et al.*, 2008). For example, a dramatic decline in spatfall of cultured oysters (*Crassostrea gigas*) in the Bay of Arachon between 1975 and 1982 led researchers to discover the extreme toxicity of tributyltin (TbT) to oysters and other invertebrates (Alzieu, 2000). Despite the subsequent developments in legislation, monitoring, and effluent treatment technologies, potential ecological effects of trace metals such as cadmium may be pertinent to aquatic life (Kannan *et al.*, 2006; Reynders *et al.*, 2008; Rosa *et al.*, 2008) and human health (Grant *et al.*, 2008) in the future. This may be because trace metal pollution seems to occur when regions increase their levels of technological or agricultural output. There is also a belief that trace metal pollution can affect remote locations away from the source (Nriagu, 1990; Dietz *et al.*, 1998). In a number of cases, exposure to heavy metals in non-industrial ecosystems has been recorded in humans (Nriagu, 1988; Järup *et al.*, 2000), marine flora such as *Fucus* (Karlsson and Eklund, 2004) and New Zealand pelagic seabirds (Nicholson and Osborn, 1983).

1.5 The relevance of copper

Copper is one of the trace elements which is essential to all life (Hart *et al.*, 1928) but which has been suspected to cause pathological effects (USEPA, 2008) and mass mortalities of aquatic species such as oysters and abalone (Orton, 1923; Martin *et al.*, 1977; Hung *et al.*, 1995). Copper based compounds are used in forestry, crop and livestock production and in heavy industry. As a result, this element has become one of the most important metal contaminants globally. Abuse of copper in agriculture has destroyed thousands of hectares of once productive agricultural land in Costa Rica (Thrupp, 1991).

The risk of contamination of coastal waters with copper increased in importance after tri-butyl-tin (TbT) was recognized as an endocrine disruptor and was subsequently banned for use on pleasure crafts in 1982 in Europe (Claisse and Alzieu, 1993). Since that first use of the precautionary principle, the background levels of copper have been increasing at

various locations in the US (Stephenson and Leonard, 1994), UK (Beaumont and Tinch, 2004), China, Italy (Munari and Mistri, 2007), Taiwan (Chao *et al.*, 2005) and Sweden (Kemikalieinspektionen, 2006). This was because copper based antifoulants were seen as the only logical alternative to TbT. Sweden subsequently banned the use of copper antifoulant paints on small pleasure craft. This ban was specifically implemented to protect beds of *Fucus* species from recurrence of local extinctions which had occurred in the Baltic in the 1970s. Even with the ban of use of copper on pleasure crafts, the use of copper based antifoulant boat paints on other types of vessels has been harmful to *Fucus* in the Bullandö marina in Sweden (Kemikalieinspektionen, 2006). Background levels of copper have also doubled in the Northern Adriatic Sea where laboratory exposures have shown significant effects of this metal on the physiology of clams (Munari and Mistri, 2007). Environmental effects have also been observed in North America where copper concentrations of 40 $\mu g\ L^{-1}$ seawater have reduced the number of phytoplankton genera in military boatyards from twelve to four (Valkirs *et al.*, 1994). Also, fish from copper contaminated sites were found with internal and external tumours (USEPA, 2008).

The current literature shows that copper is ranked as the most important metal contaminant in US waters where 41 estuaries have been classified as impaired specifically because of this metal (Arnold *et al.*, 2006). Copper contamination is also important in the UK and Sweden (Beaumont and Tinch, 2004; Kemikalieinspektionen, 2006). The concern over this contaminant is because it affects the reproductive physiology of flora such as *Fucus* which forms important nursery habitats for fish and invertebrates in the Baltic (Andersson and Kautsky, 1996). Copper can also affect mussels which can colonize new locations and create habitats for other animals in intertidal zones (Manley, 1983; Jones *et al.*, 1994; Grout and Levings, 2001).

One of the largest emitters of copper into the aquatic environment is the shipbuilding and ship maintenance industry (Dietz *et al.*, 1998). Additional sources include factories and power plants which use copper heat exchangers to lower the temperature of condenser water. Emissions from these sources are important because they have previously killed wildlife due to acute exposures (Martin *et al.*, 1977; Eisler, 1998). However, most of the documented ecological effects of copper have been attributed to chronic exposures often spanning decades (Andersson and Kautsky, 1996; Nriagu *et al.*, 1998; Beaumont and Tinch, 2004; USEPA, 2008). This tendency for long term versus short term effects of

copper on oysters and fish may be because accumulation of copper in tissue in the short term is regulated by uptake sites which ensure that adequate concentrations of the essential trace element are available under varying environmental conditions (Wright and Zamuda, 1987).

1.6 The relevance of cadmium

Another trace metal which has caused effects in humans as well as marine wildlife is cadmium. The importance of cadmium reached its climax in 1968 when the Japanese Ministry of Health and Welfare agreed that cadmium was the cause of *Itai Itai* disease which had caused 40-100% mortality in victims who resided in cadmium polluted areas in Japan (Hellstrom *et al.*, 2001). Although the incidence of *Itai Itai* disease has virtually disappeared, cadmium is believed to be affecting human heath at concentrations lower than previously believed (Hellstrom *et al.*, 2001). Effects of cadmium at low levels are also believed to be harmful to wildlife including Arctic birds (Dietz *et al.*, 1998) and sea otters (Kannan *et al.*, 2006) which have experienced increased incidence of disease and emaciation. Sea birds with slow growth rates and long lifespans such as prions *Pachyptila* and penguins *Eudyptula* in New Zealand waters have been shown to accumulate cadmium at concentrations higher than in terrestrial birds, a finding which is said to reflect the diet of these birds (Lock *et al.*, 1992; Szefer *et al.*, 1993). Seabirds such as albatrosses (*Diomedea*) in South Africa also accumulate cadmium at levels which would be toxic to humans (Muirhead and Furness, 1988).

The ecological and public health implications of regionally distributed cadmium in natural ecosystems have prompted various regional organizations such as Oslo-Paris Convention for the Prevention of Marine Pollution from Land-based Sources to the North-East Atlantic (OSPAR) to develop policy papers on this metal (OSPAR, 2004). The actions developed by this convention include monitoring of levels of cadmium in bivalves as well as financial incentives to reduce or replace the cadmium used in industry (OSPAR, 2004). Also, a conclusion of the Swedish osteoporosis, cadmium as a risk factor (OSCAR) report was that measures to reduce the exposure of the general population to cadmium were needed (Järup *et al.*, 2000). This may also be relevant for mussels because these animals accumulate cadmium by passive transport or simple diffusion across the gills (Carpene and George, 1981).

1.7 Risk assessment of trace metals

The use of small animals as biological monitors was first envisioned for the purpose of the protection of human life in coal mines subject to elevated carbon monoxide levels. In this early example, the potential value of biological sentinels was advocated by Haldane (Haldane, 1895) who used mice as a warning signal of carbon monoxide to miners. Mice and canaries subsequently became standard indicators of carbon monoxide in coal mines in the 20th century.

With the increase in use of agricultural pesticides during the Green Revolution, killifish (*Fundulus heteroclitus*) were used as some of the first indicators of the presence of trace metals and other toxic compounds in aquatic environments (Eisler, 1967). Mussels were also used as early as 1968 (Holden, 1973) but the first programme to focus on chemical residues in mussels was the mussel watch sponsored by the United States Environmental Protection Agency (Goldberg, 1975). Laboratory exposures were used to calibrate the concentration of toxins determined for mussels collected from the field (Fischer, 1988; Engel and Fowler, 1988).

The early laboratory methods used to calibrate tissue concentration of toxins were static exposure systems. These methods required considerable handling of the exposed animals which were often stressed (Ahsanullah, 1974). As a result, gravity fed flow through systems were designed which allowed for continuous renewal of exposure water without disturbance to the test animals (USEPA, 2000; Rand *et al.*, 2003). Flow through systems were said to be better because they were not expensive to construct and facilitated the use of natural waters in exposure experiments (Charles and Newell, 1997). The particulate organic matter present in natural seawater is said to contribute to the nutrition of bivalve filter feeders held in laboratory conditions (Siebers and Winkler, 1984). Additionally, flow through systems help to maintain higher dissolved oxygen and lower levels of metabolites such as ammonia which can affect the survival and physiology of test animals (Benoit *et al.*, 1993). Flow through systems allow for more treatment combinations, require less time for renewals of exposure waters and are important when gentle renewals of exposure water are desired. Flow through systems also allow for use of self starting siphons for removal of the spent exposure water (Benoit *et al.*, 1993).

Along with the development of laboratory exposure systems, field techniques were developed to identify aquatic resources which become affected by DDT and mercuric pesticides (Farrington *et al.*, 1983; Lauenstein and Daskalakis, 1998). Work in North America concentrated on correlating the level of trace metals in the tissues of mussels with ambient levels in sediments and the water column. In Europe, the biomonitoring strategies included using physiological measurements on the mussels. These have proven useful for detection of various types of inorganic contaminants including trace metals in marine environments (Widdows and Johnson, 1988).

1.8 SfG and other bio-indicator techniques

Some of the major incidents of aquatic pollution were discovered because of impaired reproduction in keystone species (Bryan *et al.*, 1986). These incidents occurred because effects of toxicants such as TbT cascaded undetected from sub-organismal levels to organismal, population and community levels which were eventually recognised by ecologists. To guard against future unprecedented ecological dangers, a mechanistic framework for environmental monitoring at suborganismal, organismal and population levels has been developed. Sub-organismal biomarkers include metallothionein expression, and acetyl cholinesterase activity. Organismal techniques have included behavioural assays and condition indices. Environmental research in Europe has included suborganismal techniques including cholinesterase activity and glycogen levels in bivalves (Monserrat *et al.*, 2006). Histopathological methods include techniques to visualize trace metals in tissue (Ros *et al.*, 2000). Organismal methods used have included histopathological techniques such as autometellography.

In addition to these methods, an organismal level technique developed to monitor sessile bivalves in Europe, North America and Oceania is Scope for Growth (SfG) (Martin *et al.*, 1984; Widdows and Johnson, 1988; Bayne *et al.*, 1997; Resgalla *et al.*, 2007b). SfG is an important biomarker because it can integrate the effects of various pollutants on sub-organismal responses like gill function and predict fitness responses such as energy acquisition, survival and fecundity which are important at the community level (Tasman *et al.*, 2004). Scope for Growth (SfG) is a numeric calculation of the difference between the energy acquired from feeding and the expenditure of energy on respiration and excretion

(Widdows and Staff, 1997). The Scope for Growth (SfG) of bivalves such as mussels is calculated using the equation:

$$SfG = C - (R + U)$$

where: C = Energy ($J\ g^{-1}\ h^{-1}$) acquired from feed particles cleared from the seawater

R = Energy ($J\ g^{-1}\ h^{-1}$) lost from respiration

U = Energy ($J\ g^{-1}\ h^{-1}$) lost from urine

Data are derived for this calculation by measuring the rate of clearance of food particles from filtered seawater and converting this into energy equivalency. Clearance rate is a measure of the volume of water cleared of suspended particles of diameter > 4 μm per hour. *Tetraselmis* species which measure > 5 μm in diameter are preferred (Widdows and Staff, 2005) because a higher percentage of these species compared to smaller species such as *Isochrysis* are retained by the bivalve gill filter feeding mechanism.

The energetic expenditure due to respiration is determined by flow through or closed box respirometry. Excretion is often determined using colorimetric determination of ammonia (Solorzano, 1969a) produced over a known period. The oxygen consumption and excretion data are converted to energetic equivalents and subtracted from the total energy acquired. Although SfG is used primarily in environmental protection programmes, the technique is also used to a limited extent for ecological and aquaculture research (Table 1.2). Few studies have utilized SfG to compare the physiology of farm and intertidal mussels. In fact, only one study has been found which has compared the SfG of farm and wild mussels of the same species (Labarta *et al.*, 1997).

Table 1.2: Studies which have used SfG for environmental (E), aquaculture (A) and ecological (C) studies.

Type	Relationship established to	Reference
E	Low SfG with low salinity	(Sarà *et al.*, 2008)
E	Low SfG of *Anadara trapezia* related to Cd & Pb of surfacial sediments	(Burt *et al.*, 2007)
C	Low SfG for *Cerastoderma edule* in winter and high values for spring and summer	(Ibarrola *et al.*, (In Press))
E	SfG and glutathione peroxidase of *M. galloprovincialis* on exposure to Ni, Cr. Fe	(Tsangaris *et al.*, 2007)
A	High SfG *Argopecten nucleus* associated with *Isochrysis* algal feeds	(Velasco, 2007)
A	High salinity increased SfG for *Argopecten purpuratus* but high NH_4^+ lowered SfG.	(Soria *et al.*, 2007)
C	Higher SfG for *Perna perna* in summer	(Resgalla *et al.*, 2007b)
E	SfG of *M. edulis* reduced in low salinity and petrol	(Prevodnik *et al.*, 2007)
E/A	SfG *Tapes philippinarum* reduced by 10 µg Cu L^{-1}	(Munari and Mistri, 2007)
E	Lowest SfG of *M. edulis* at 26°C compared to 6 °C, 16 °C and 13 °C.	(Mubiana and Blust, 2007)
C	SfG of *P. canaliculus* decreased with decrease availability of seston	(Helson and Gardner, 2007)
E	SfG of *P. viridis* negatively correlated with DDT	(Shuhong *et al.*, 2005)
C	SfG of *Spisula subtruncata* high in summer and low in winter	(Rueda and Smaal, 2004)
C	Higher SfG of *Mulinia edulis Mytilus chilensis* at seston >100 mg L^{-1}	(Velasco and Navarro, 2003)
A	SfG of *Crassostrea belcheri* declined with exposure to Cu	(Elfwing and Tedengren, 2002a)
C	SfG of *Argopecten purpuratus* increased with lipid content of diet	(Navarro *et al.*, 2000)
E	SfG of *Ruditapes decussatus* declined by 80% on exposure to 10 µg Cu L^{-1}	(Sobral and Widdows, 1997b)
E	SfG of *Ruditapes decussatus* declined by 90% under anoxic conditions	(Sobral and Widdows, 1997a)
A	SfG of intertidal *M. galloprovincialis* responded to laboratory conditions better than raft mussels	(Labarta *et al.*, 1997)

1.9 Inter laboratory standardization of methods

Because a number of laboratories conduct studies which incorporate various aspects of the SfG methodology, it was recognized early that there was a need for standardization of techniques at national and international levels (Bayne *et al.*, 1988). The need for inter-

laboratory calibration of methods is still relevant because the design of field experiments utilizing SfG continues to be refined.

For example, a study comparing the SfG of mussels from pristine and contaminated sites in Sydney Harbour showed that controls needed to be transplanted from unpolluted sites back to unpolluted sites to control for background sources of unknown toxicants as well as to control for the handling of transplants. The results of that study were that sources of disturbance or stress in the control treatments had been overlooked and caused low scope for growth in control mussels transferred from the pristine site back to the pristine sites (Honkoop *et al.*, 2003).

Surprising results have also occurred when back transplanted controls were not utilized. For example, results of mussel transplantations conducted in Wellington (Anderlini, 1992) showed that SfG correlated negatively with copper but not cadmium in tissue of the transplanted mussels. Also, SfG was higher in sewage waters containing higher concentrations of trace metals. Another example of lack of correlation between biomarkers and contaminants was with studies on the mussel *Mytilus trossuls* exposed to crude oil from the Exxon Valdez oil spill. Three years after the spill, mussels from a contaminated site showed no impairment in byssal thread production, condition index, clearance rate or glycogen content (Thomas *et al.*, 1999) in comparison to mussels from an un-impacted site. These results contrast with other studies in which mussels exposed to chronic low levels of hydrocarbons in the North Sea showed clear responses in SfG (Widdows *et al.*, 1995).

The lack of significant effects of contaminants on SfG or other measures of stress in a number of studies can partly be explained because mussels in natural ecosystems are subject to numerous natural stresses in addition to stresses caused by contaminants (Hellou and Law, 2003). These stresses include obvious environmental variables such as water temperature and salinity as well as variables such as wind intensity, turbidity and wave height (Resgalla *et al.*, 2007b) and even stress caused by the experimenter (Carefoot, 1990b). The effects of some of the un-identified or uncontrolled stresses could yield results similar to end stage stress caused by polluted environments if they are not controlled with the appropriate experimental protocols. As a result, the identification of naturally occurring factors which lead to experimental or environmental stress and mortality of bivalves in the field is the subject of international research (Rajagopal *et al.*,

2005) as well as research in New Zealand (Carton *et al.*, 2007). Such studies have resulted in development of knowledge of bivalve behaviour as individuals as well communities (Filgueira *et al.*, 2006; Elliott *et al.*, 2008).

1.10 Sources of variability of SfG

The term stress refers to all deviations from steady-state functions which predispose mussels to further deviations (Bayne *et al.*, 1976a). SfG is a biomarker which has been developed to quantify pollution induced stress (Widdows *et al.*, 1995). However, even before contaminant induced stress becomes evident, bivalves have to accommodate environmental conditions which fluctuate between optimal and sub-optimal on a daily or hourly basis. These deviations may be caused by a number of environmental factors which interact with the intrinsic factors such as the weight of tissue of mussels.

Intrinsic factors

Size of mussels

Size measured as shell length or dry tissue weight has been shown to affect respiration and other components of SfG of various mytilid mussels and other bivalves (L.Bayne *et al.*, 1976; Navarro, 1988; James *et al.*, 2001; Resgalla *et al.*, 2006). The relationship between size and respiration or clearance rates in mussels has been described as an allometric function $Y = aX^b$ where Y is the physiological rate measured, a is the rate of a 1 gram animal and b is an allometric exponent which scales the observed physiological index measured with the size of the animal. The value of this exponent is said to be vary between 2/3 (Ibarrola *et al.*, 2008) and 3/4 (Hulbert and Else, 2004) but values lower than this have been recorded for *P. canaliculus* (James *et al.*, 2001). The variability in physiological rates measured can be addressed by using mussels of a standard weight but weights and the allometric function vary seasonally (James *et al.*, 2001).

Origin of mussels

A number of issues arise when research on the physiology of field collected bivalves such as *P. canaliculus* is undertaken for the purpose of identification of threats to the

environment or threats to the cultured species. Previous research has shown that the SfG of cultured mussels reverse transplanted between two Scottish lochs was different from SfG of the resident mussels at 15 days after transplantation but were similar to the native mussels after 4.5 months. This showed that the physiological rates recorded in mussels resident in an area are likely to be due to environmental factors (Okumus and Stirling, 1994). Other research showed that when wild and raft-cultured mussels were transferred to laboratory conditions, the SfG of wild mussels increased by 39.4% while SfG of farm mussels increased by 20.2 % over 15 days. However, initial differences were reduced by less than 10% after 15 days indicating the persistence of ecological memory and energetic advantages in the cultured mussels (Labarta *et al.*, 1997). Differences in growth even within culture rafts were found to reflect the differences in the food resources available to individuals. These previous studies suggest that, upon evaluation of the physiological indices of mussels, attention has to be paid to the environmental conditions to which the sentinels were previously subjected.

Environmental factors

Salinity

Salinity is one of the variables which could constitute an important source of uncontrolled variance in physiological experiments on marine animals such as mussels (Monserrat *et al.*, 2006). The effects of salinity on physiology of mussels are usually difficult to investigate under field conditions because decreases in salinity often occur with a parallel influx of nutrients from runoff. Reduced salinity may affect animals directly or indirectly as a result of increased algal production which may cause higher clearance rates (Riisgård *et al.*, 2003).

The direct effects of salinity on physiological rates are more thoroughly studied. For example, although the mussel *Choromytilus chorus* displayed high SfG at salinities above 30 ppt, the SfG was not affected by reduced salinity between 24 ppt and 30 ppt (Navarro, 1988). The SfG of this species became negative at 16 ppt which affected larger mussels to a greater degree than smaller mussels (Navarro, 1988). In addition to being directly effected by salinity, different populations of bivalves may respond differently to changes in salinity (Navarro and Gonzalez, 1998). For example, an experiment was conducted in

which marine mussels were transferred from oceanic sites (28-32 ppt) and sites subject to periodic riverine influences respectively (6-32 ppt). The mussels were then exposed to a range of salinities under laboratory conditions. Results showed that the mussels previously exposed to riverine influences showed significantly higher clearance rates compared to mussels sourced from the marine sites (Blackmore and Wang, 2003). This suggests that mussels subject to more variable environments were able to adapt to different salinities better than mussels which had never experienced fluctuations. This is relevant to the current study because it is possible that intertidal mussels and farm mussels differ in their abilities to maintain clearance rates at low salinities.

There are only few laboratory studies which document physiological effects of salinity on New Zealand bivalves (Marsden, 2004). However, field studies have shown that lower salinity at monitoring stations such as Matiu-Somes Island in Wellington Harbour contained mussels with higher gonadal mass and condition indices than sites with higher salinity (Lachowicz, 2005). In other studies, effects of low salinity on condition of *Austrovenus stutchburyi* were secondary to the effects of low food (Marsden, 2004). This is because at low salinity, species such as *Austrovenus stutchburyi* made maximal use of the high tide by feeding primarily during this time (Marsden, 2004). This shows that intertidal marine species may have significant capacity to cope with changes in salinity. However, in polluted environments, sessile marine species also have to cope with changes in the speciation of essential and nonessential trace elements which occur when salinity changes. The indirect effects of reduced salinity arise because at low salinity, there are less carbonate and chloride ions in natural waters to complex with toxic metals such as copper or cadmium. The complexes which form between toxic metals and carbonate or chloride ions in water are generally less toxic (Neff, 2002) than the metal ions not in a complex.

These indirect effects of changes in salinity include differences in the availability of essential nutrients such as iron as well as differences in toxicities of non essential elements which can affect bivalve filter feeders under natural physiological stress (Mubiana and Blust, 2007). In accordance with this, metals such as cadmium or copper cause higher mortality to aquatic animals in lower salinity (McLusky *et al.*, 1986). However, few studies have demonstrated the combined effects of reduced salinity and toxic trace metals on physiological indices of bivalve sentinels.

Temperature

The relationship between temperature on growth rate of mussels is described as a positive relationship within the range of the mussel (Blanchette and Gaines, 2007). It is also recognized that mussels in deeper colder waters attain larger size probably as a result of lower metabolic losses (MacDonald and Thompson, 1985). The effect of temperature on the growth rate of *P. canaliculus* has been established based on early growth trials conducted in the North and South Islands of New Zealand (Hickman, 1979). This early study showed that growth of *P. canaliculus* over 365 days at Waihi was 29 g versus growth of 7.2 g at Bluff (Hickman, 1979). The factors which cause such differences included differences in clearance, respiration and absorption efficiency. For example, filtration rate of *P. canaliculus* was higher at 18°C versus 15°C and 12°C (Waite, 1989). Also, *P. canaliculus* displayed higher respiration at 15°C and 20°C compared to 10°C but aerial respiration rates were depressed at the higher temperatures (Marsden and Weatherhead, 1998). Temperature can easily be controlled in most research settings and pose no major problems for laboratory based research which investigate other variables.

Ration

Mussels feed by creating a current over this gills and removing food particles from the water. The volume of water moved over the gills is referred to as the filtered water. The fraction of this water which is cleared of particles >5 μm diameter is called the clearance rate. The filtration rate usually increases with seston concentration before it declines (Navarro and Widdows, 1997). Clearance rate on the other hand usually declines with increasing seston concentration (Richoux and Thompson, 2001). This is because mussels are said to be able to decrease the velocity of delivery of particles to the ventral groove in the gills and prevent overloading of the feeding mechanism (Richoux and Thompson, 2001). As a result, ingestion in mussels is said to fit an exponential curve which does not decline at high concentrations of seston (Babarro *et al.*, 2000a).

This feeding response may present two sources of variability in measuring SfG of *P. canaliculus*. Firstly, the decrease delivery of particles to the ventral groove at high seston concentrations may have been the cause of the decrease in clearance of *P. canaliculus* at high total particulate matter (Hawkins *et al.*, 1999). In addition, studies on the feeding and

energetics of *Mytilus edulis* have shown that physiology of this species is sensitive to low levels of feeding (Widdows, 1978b). This may be because although it takes considerable lengths of time (Widdows, 1978a) for starved mussels to die, the period of starvation represents a period of energetic expenditure for minimal gains. When no feed was provided for *Mytilus edulis*, the filtration rate was decreased to low levels but these mussels were able to increase this considerably within one hour (Thompson and Bayne, 1972).

1.11 Why study SfG of mussels?

Starting from early works on the energetics of filter feeders (Thompson and Bayne, 1972; Bayne, 1973b; Bayne *et al.*, 1976a), numerous studies (Table 1.2) have shown that pollution affected the SfG of mussels such as *Mytilus edulis* at remote locations. These studies include the North Sea UK east coast survey of 1990-1991 and the Irish Sea survey of 1996-1997 (OSPAR, 2000). These studies developed standard operating procedures to minimise the effects of stress and disturbance on the mussels during the experiments. The standard operating procedure included use of high quality offshore 33 ppt salinity seawater, a standard temperature of 15^{o}C, and feeding of mussels with a standard ration of *Isochrysis galbana* (Widdows *et al.*, 1995). Mussels were sourced from field sites and shipped by courier in insulated boxes without water and they were allowed to recover for 24 hours prior to evaluation of clearance, respiration, and excretion using high quality seawater in the experimental apparatus. This method showed that field mussels reflected stresses which they exhibited in polluted environments. SfG has subsequently been used as an environmental tool in Asia, the Mediterranean and Oceania.

In addition to environmental studies, studies on the energetics of mussels have been used for aquaculture purposes (Newell *et al.*, 2001). Cross disciplinary research between ecology and aquaculture of mussels also suggest that farmed mussels could be valuable biomonitors of environmental health because of to their immediate commercial value (Medeiros *et al.*, ; Viarengo and Canesi, 1991). However, wild mussels were reported to provide a better snapshot of cumulative exposure to toxicants while farm mussels only showed the effects of recent exposures (Bolognesi *et al.*, 2004). This may be because origin of mussels is an important source of physiology adaptation which intertidal and farm mussels may retain (Babarro *et al.*, 2000b). For example, minor differences in the vertical

niche from which mussels are collected from may have major effects on the respiration rates of intertidal mussels (Widdows and Shick, 1985; Shick *et al.*, 1988; Marsden and Weatherhead, 1999). The differences in physiological adaptations to natural and anthropogenic stressors are important to evaluate because these could identify phenotypes of mussels such as *P. canaliculus* which are more vulnerable to environmental change.

1.12 Why study SfG of *Perna canaliculus?*

SfG of *P. canaliculus* has not been widely studied but the first study in 1987 in Wellinton Harbour showed a strong negative relationship (R = -0.837) between mussel tissue level of copper and SfG. On the other hand, a positive relationship (R = 0.755) was found between cadmium levels in the tissue and SfG of the mussels (Anderlini, 1992). In another study SfG was found to be similarly low for *P. canaliculus* collected from rafts in Mahanga Bay and from wharf pilings in Days Bay in Wellington (Gardner and Thompson, 2001). The reason for the negative SfG was because of low seston levels. Subsequent research in New Zealand suggests that SfG may overestimate the energetic balance of intertidal *P. canaliculus* (Helson and Gardner, 2007). Additionally, considerable variation within vertical height of a mussel bed in Canterbury suggested that SfG of this species may be highly variable (Weatherhead, 1993). Such studies imply that certain aspects of the SfG methodology may need further refinement to be considered a viable tool for environmental protection of New Zealand's coastal resources.

In the future, detection of physiological differences between farm or naturally occurring intertidal mussels challenged with natural stressors or contaminants could be a valuable application of the SfG methodology for environmental protection and aquaculture in New Zealand.

1.13 Objectives of the thesis

The general aim of this research was to determine if farm or intertidal *P. canaliculus* mussels showed different physiological responses to copper and cadmium in full strength (34 ppt) and half strength (17 ppt) seawater. This was in order to conclude whether SfG would be applicable as a tool for environmental assessment in dynamic environments

subject to large changes in salinity. The null hypotheses tested were that mussels exposed to seawater treated with copper, cadmium, or reduced salinity would show no differences in clearance, respiration, excretion, SfG and condition index compared to mussels exposed to natural seawater.

1.14 Organization of the thesis

The thesis comprises one overall methods chapter (Chapter 2) and five results chapters (Chapters 3-7). The results chapters report 17 experiments which were conducted to achieve the goal of identifying how mussels of different origins differ in their response to contamination of water.

Chapter three investigated the effects of different maintenance, feeding, time of day, dry tissue mass and origin of mussels on the metabolic rates of mussels.

Chapter four reports on four experiments. The first experiment described the effects of 0 μg Cu L^{-1} - 100 μg Cu L^{-1} on the clearance and respiration rates of intertidal mussels. Experiment two and three describe the effects of 0 μg Cu L^{-1} - 1,000 μg Cu L^{-1} on the clearance, respiration and condition of farm and intertidal mussels. Experiment four describes the effect of 0 μg Cu L^{-1} - 500 μg Cu L^{-1}on SfG of intertidal mussels.

Chapter five reports the effects of 0 μg Cd L^{-1}- 99 μg Cd L^{-1} on SfG of farm and intertidal mussels. Chapter five also reports the effects of 0 μg Cd L^{-1}, 500 μg Cd L^{-1}, 1,000 μg Cd L^{-1} and 1,500 μg Cd L^{-1} on intertidal specimens.

Chapter six reports the effects of combinations of 0 μg Cd L^{-1} and 1,500 μg Cd L^{-1} with 34 ppt and 17 ppt seawater on farm and intertidal mussels.

Chapter seven reports the effects of combinations of 0 μg Cu L^{-1} and 100 μg Cu L^{-1} with 17 ppt and 34 ppt salinity seawater.

Chapter eight compares the rates determined for the controls in the context of the current literature on *Mytilus* and *Perna* mussels. This chapter compares the differences in physiological responses of farm and intertidal mussels challenged with reduced salinity reported in chapters six and seven. Chapter eight also compares the physiological effects of the metals on farm and intertidal mussels.

Chapter 2 Materials and Methods

2.1 Collection

The mussels used in the experiments described in this thesis were sourced at coastal locations south-east of Christchurch (Figure 2.1). The dominant ocean current in this area moves water northward (Gardner *et al.*, 1996) so the sites were not likely to be affected by municipal effluents from the Avonhead-Heathcote estuary which is the major source of anthropogenic contaminants and nutrient enrichment into Pegasus Bay.

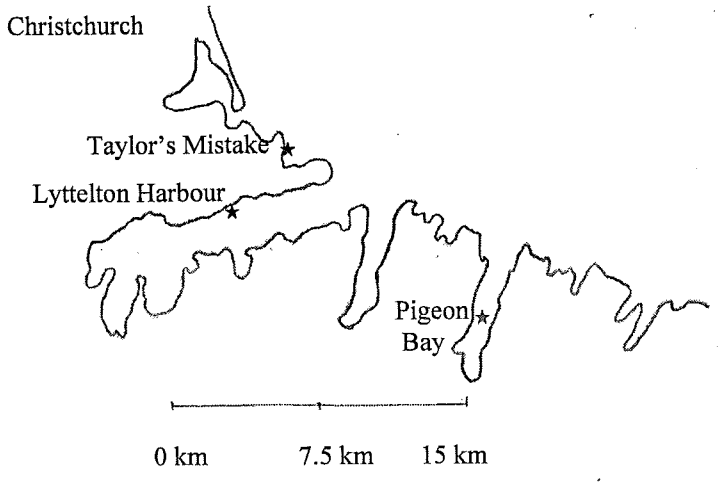


Figure 2.1: Map of area near to Christchurch from which seawater, intertidal and farm mussels were sourced.

All the intertidal mussels used in this research were collected north of the embayment at Taylors Mistake (Figure 2.1). Clumps of mussels were removed as a group where mussels were attached to each other in layers above larger mussels which were directly attached to the rock substrate. The clumps may have contained *Mytilus galloprovincialis* and *Aulacomya maoriana* in addition to the *P. canaliculus* sought. After each clump was gathered, the larger *P. canaliculus* were removed from the clumps and placed, along with the *Mytilus galloprovincialis* and *Aulacomya maoriana* back on the rocks. The small *P. canaliculus* which remained were separated from each other and the shell lengths measured with rulers. This allowed for selection of mussels in the length range of 5.9-7 cm using calipers.

The farm mussels used in other experiments were sourced from a farm at Pigeon Bay and delivered in styrofoam bins via same day courier to the University of Canterbury School of Biological Sciences (SBS). These locations for collection of mussels and seawater (Lyttelton Harbour) were selected to represent sites where naturally occurring intertidal mussels and farmed *P. canaliculus* mussels were occupying sites which had minimal previous exposures to contaminants.

2.2 Maintenance

Mussels collected from the coastal sites were transported in buckets to the SBS aquarium and placed in 30 L plastic containers which were part of a recirculating seawater system. Seawater in this system was kept at 13°C –15 °C and at salinity of 33 ppt-35 ppt. Each 30 L tank was supplied via a valved plastic tube. The outlet was a PVC standpipe which was meshed to prevent escape of the contents of each tank. Lighting in the aquarium was in a 12:12 hour light:dark cycle. Mussels that were placed in this system usually became attached to the bottom and sides of the aquarium tanks and their byssus threads had to be severed at the start of the experiments. Feed application to the maintenance tanks was also a problem initially because feed and faeces also settled at the bottom of the tanks and caused the development of anoxic conditions. For this reason, all tanks were removed from the system and cleaned when each set of fresh experimental animals arrived. Water for maintenance and for all the experiments was collected from Lyttelton Harbour and transported on a weekly basis to the SBS in a stainless steel container.

2.3 Attachment

Perna canaliculus mussels tend to attach themselves to artificial or natural substrates if moved to a new location. The experiments in this research involved measurement of three indices which were generally difficult to measure in the same containers.

Because mussels needed to be moved from the metal exposure tanks to the vessels in which clearance and oxygen consumption were measured, a system was devised whereby mussels could be moved without having to touch or cut byssus threads attaching the mussels to their substrate. The method of attachment which was developed was designed to ensure that mussels would form easily manipulated clumps of only four or five mussels. When the mussels were separated, individual mussels would still be attached to a substrate and would be easily handled for determination of clearance rate. The systems also allowed for rapid and less stressful transfer of the mussels to the respirometers for determination of oxygen consumption after the clearance rate experiments were finished.

This use of mussels attached to perspex plates was only initiated after the experiments reported in chapter four were conducted. All experiments described in chapters five, six and seven were conducted on mussels which had been attached to 5 cm x 5 cm plastic plates (Figure 2.2) using cyanoacrylate glue four or five days prior to the start of the metal exposures (Figure 2.2). Each mussel from the exposure tanks could be checked each day for mortality without having to touch the mussels directly or disturbing the byssus. This also facilitated quick removal of mussels from the exposure tanks. In addition, this system minimized the risk of cross contamination of cadmium or copper between tanks because the mussels were lifted out of the exposure using the string which was held outside the water by ropes tied across the top of the exposure containers (Figure 2.7 and 2.8). As a result, there was no direct contact between the exposure water and the gloved hand of the experimenter when the mussels were checked for mortality each day. This system also minimized the risk of exposure to cadmium or copper by the experimenter because there was no need to insert hands into the exposure water to manipulate the mussels.

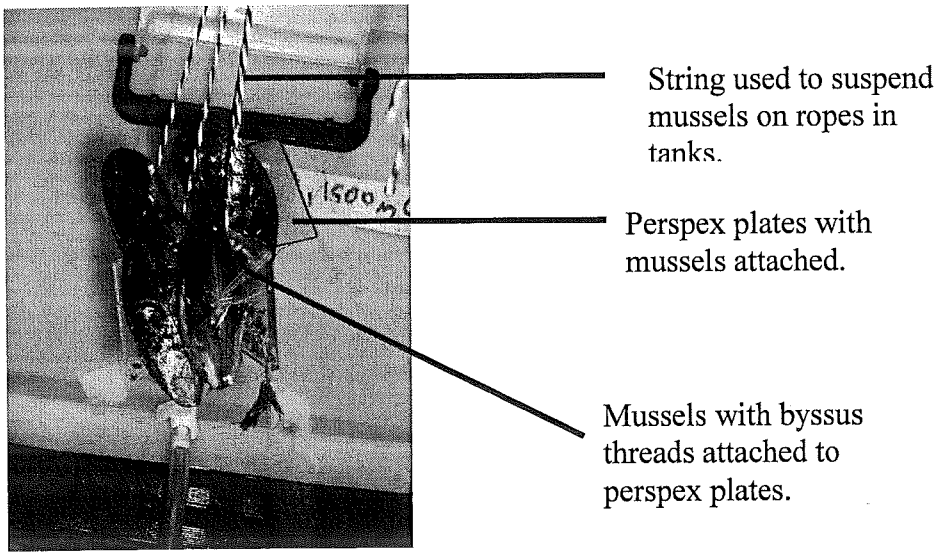
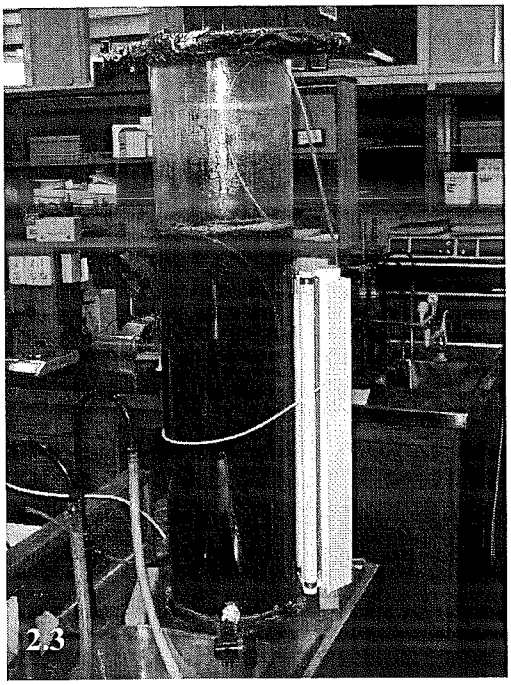


Figure 2.2: Mussels with byssus attached to plastic plates.

2.4 Culture of algal food

Mussels were provided with food comprising a frozen paste of *Tetraselmis chuii* which was thawed before feeding to the mussels (Figure 2.6). Concentrated natural feed was used because, in previous research, it had been shown that addition of live cultures at concentrations derived directly from actively growing cultures were not enough to preserve mussel function. *Tetraselmis chuii* was chosen as the feed because this species had been previously shown to be filtered effectively by mytilid mussels (Widdows and Staff, 2005).



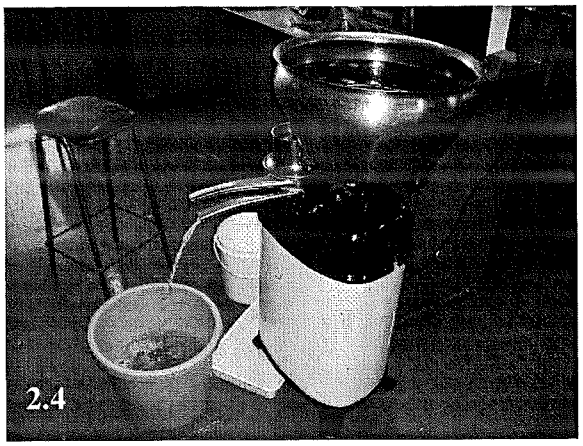
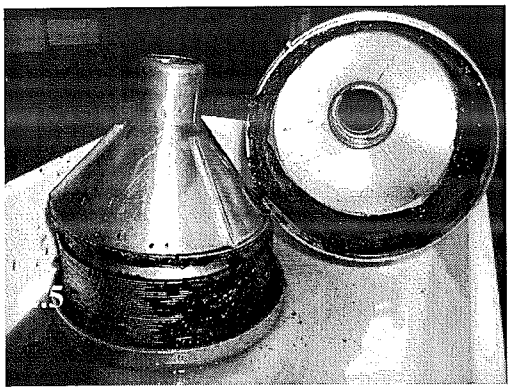


Figure 2.3: 40 L algal growth tube with *T. chuii* culture.
Figure 2.4: Alfa Laval[R] milk cream separator used to concentrate *T. chuii* for feeding to mussels.



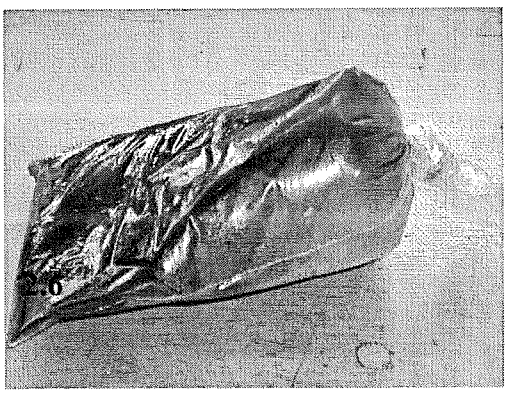


Figure 2.5: Separation discs with concentrated *T. chuii.*
Figure 2.6: Concentrated *Tetraselmis chuii* in plastic bag before thawing.

A frozen concentrate of this algal species was used because it allowed for convenience of storage and the low metabolic rate of the algae at the time of application into the exposure tanks.

This concentrate used was made by culturing *Tetraselmis chuii* in F/2 media in a 40 L algae growth tube (Figure 2.3) and four 15 L plastic carboys. The live cultures were dewatered after two weeks of culture (Figure 2.4, 2.5). Dewatering produced a paste (Figure 2.5) which was reconstituted then frozen for later feeding to the mussels as a liquid concentrated.

The dewatering of the pure culture was done by repeated centrifugation (6-10 times) of the pure cultures using an Alfa Laval^{R} milk creamer (Figure 2.4). This process produced an algal paste inside the creamer mechanism. The paste was collected by disassembling the centrifuging system and scooping out the paste with a spatula. This paste was placed in a plastic bucket and reconstituted using unfiltered seawater to 3 L. In order to estimate the dry weight of the paste, 4 ml sample of this concentrate was then diluted into 1 L distilled water. A 3.5 ml sample of this was then placed in a methacrylate cuvette which was read in a Turner Designs Aquafluor^{R} fluorometer. If the fluorescence reading was >4,000, seawater was added to dilute the algal concentrate to a concentration which would approximate 0.07 g dry *Tetraselmis chuii* per ml of concentrate. This was based on a relationship of

dry mass of algae (g ml^{-1}) = ($4e^{-05}$) (fluorometer reading) + 0.0724

This concentrate was added to each tank at a rate of 65-70 ml per tank per day to cover 5 % of body weight per mussel per day as feed.

2.5 Exposure system

The exposure system used for the experiments comprised of eight 37 L plastic containers used as header tanks and exposure aquaria (Figure 2.7). These tanks contained seawater with various combinations of salinity and copper or cadmium. Four containers served as headers from which water was gravity fed to the exposure containers a level below at a rate of 25 ml min^{-1} using plastic valves. The water displaced from the exposure tanks was shunted into collection tanks below via overflow outlets (Figure 2.8).

Figure 2.7: Three tier gravity flow through system used to conduct metal and low salinity exposures

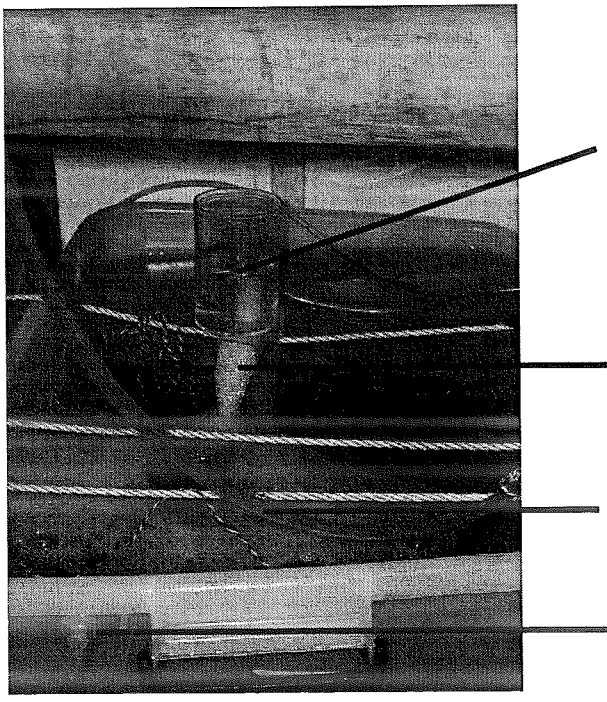


Figure 2.8: Protein skimmer used to aerate exposure water and create flow in tank

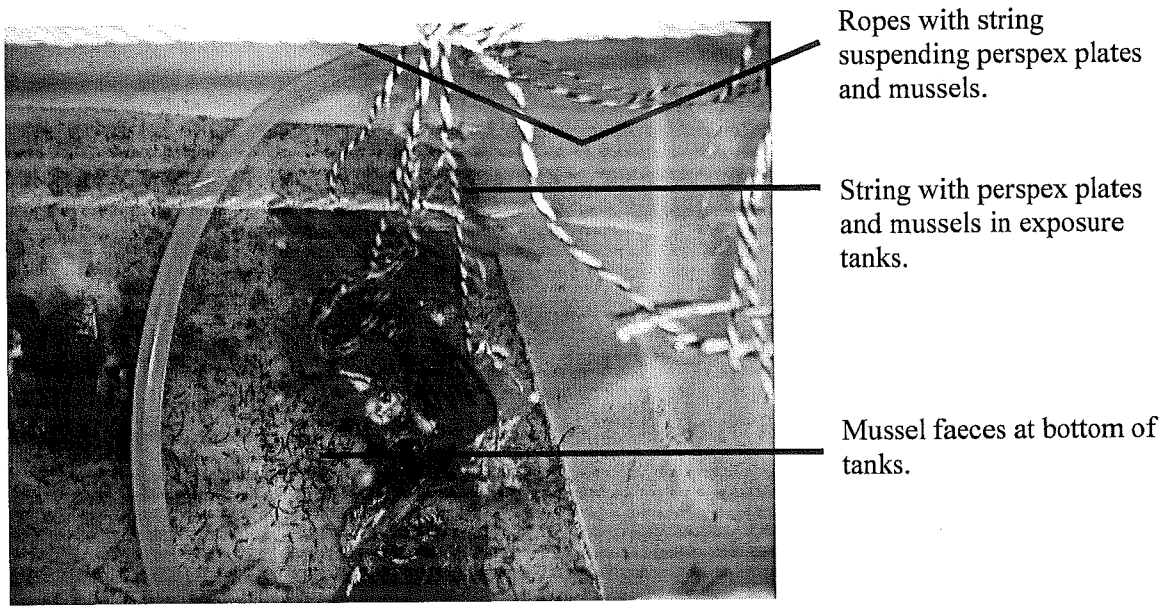


Figure 2.9: Mussels attached to plastic plates in exposure tanks.

Each exposure aquaria was fitted with a protein skimmer which provided water flow within the tanks and which also served to sequester dissolved protein collected as foam (Figure 2.8).

Three lengths of plastic rope were stretched in parallel tightly above the rim of the four exposure aquaria. These ropes were used to suspend the plastic plates with individually tagged and glued mussels (Figure 2.9). In total, 24 mussels attached were stocked per tank for each treatment concentration in order to be able to sample (n = 8) mussels after three, seven and fourteen days of exposure to the treatment combinations. The eight mussels per treatment were stocked on a staggered basis over two consecutive days. Half the mussels (n = 4) were stocked on the first day and half on the subsequent day. Each set (n = 4) of mussels was suspended separately on each rope support (Figure 2.9). Each set was labelled with a different colour string so there were two sets of mussels suspended from each rope over each tank. With this system, mussels were suspended with the siphons facing upward in the column of water. In addition to the mussels used for physiological measurements, 18 mussels were stocked to determine the glycogen, lipid and protein content of the adductor muscle and digestive glands on n = 6 mussels after three, seven and

fourteen days of exposure. At each time interval, samples of 0.1 g, 0.025-0.05 g and 0.3 g of adductor tissue were collected from each mussel. Each tissue was weighed and placed in an eppendorf tube and frozen with liquid nitrogen. In addition to the adductor tissue, the hepatopancreas of each mussel was extracted, weighed and placed in a separate eppendorf tube. These samples were then transferred to a freezer at $-18^{\circ}C$. Nine mussels were also stocked to do histological studies on a sample of n = 3 mussels after exposure to the treatments for three, seven and fourteen days of exposure. These mussels were shucked and placed in Davidsons Fixative for three days. After this period, the mussels were transferred to a solution of 70% ethanol.

2.6 Clearance rate

Clearance rate is a physiological index used to calculate an estimate of the quantity of energy which a mussel is able to acquire. The calculation is based on the relationship between the rate of removal of food particles from seawater and the calorific value of these particles. Clearance rate is defined as the volume of water cleared of suspended particles of diameter > 4 μm per hour (Widdows and Staff, 2005). Clearance was calculated by quantifying the proportion of *Tetraselmis chuii* removed from a seawater sample. This value was then used to extrapolate the quantity of seawater that had been filtered by the mussel.

In my studies, clearance rates were measured on individual mussels suspended with the siphons facing upward in 3.9 L of filtered seawater in a plastic container (Figure 2.10 (a)). Clearance rate in a total of 16 mussels plus a control was done simultaneously (Figure 2.10 (b)).

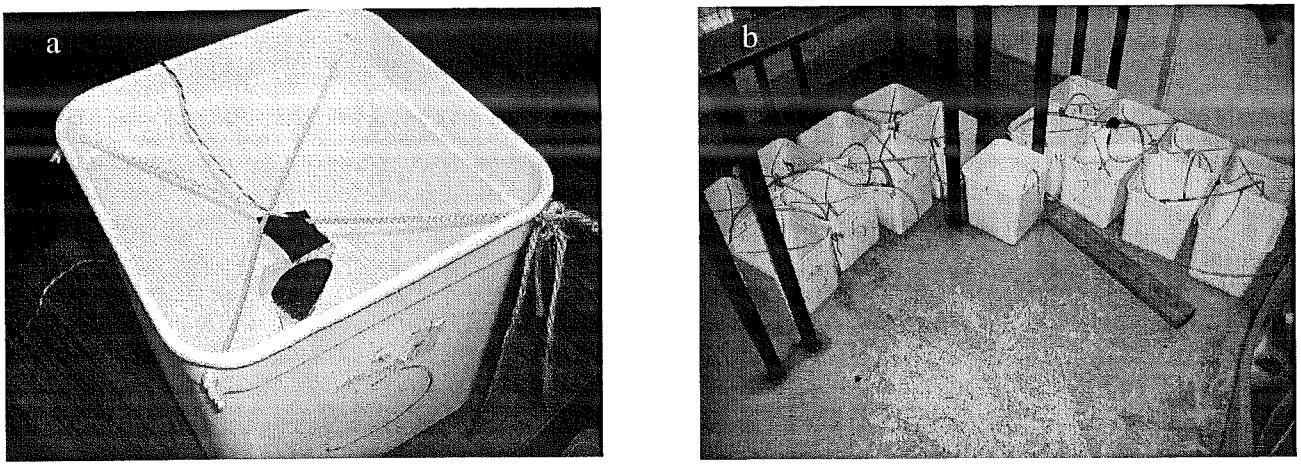


Figure 2.10: (a) Mussel suspended in 3.9 L container for measurement of clearance rate, (b) containers for measurement of clearance rate of sixteen mussels.

The seawater in the clearance rate experiments was filtered through a 0.5 μm filter. This water was then placed in the containers and gently agitated with compressed air supplied by an air-stone. The experimental mussels were suspended in each 3.9 L container, using velcro to attach the string on which the plastic plates containing the mussels were tied.

Fresh *Tetraselmis chuii* cultures were added to the control container and the concentration of the *Tetraselmis* checked to ensure that it was below 25,000 cells ml^{-1}. This corresponded to a fluorometer reading of less than 500-600 fluorometer units. The amount was then adjusted for the rest of the buckets. Mussels were allowed to settle for ten minutes before evaluation of the clearance rate was carried out every 20 minutes over a period of two hours. The clearance rate for each 20 minute interval was calculated as

$$CR\ (L\ h^{-1}) = \frac{(V) \times (\ln C_1 - \ln C_2)}{t}$$

where:

V = total volume of seawater used in the clearance experiment

C_1 = cell concentrations (ml^{-1}) at the beginning of each 20 minute interval

C_2 = cell concentration (ml $^{-1}$) at the end of the 20 minute interval

t = time interval (20 minutes) between concentration measurements

The average of four successive 20 minute intervals was calculated and the maximum values of these averages represented the clearance (L h^{-1}). This was then expressed per the dry tissue weight of the mussel to yield clearance rate (L g^{-1} h^{-1}). To calculate the SfG, the clearance rate was converted to calorific value by multiplying by constants of 9.2 J L^{-1} for the energy content of algal food and 0.45 for the absorption efficiency of the algal food (Widdows and Staff, 2005).

2.7 Respiration and excretion rates

Respiration rate

The respiration rate determined for mussels is a comprehensive approximation of the total costs which must be covered for maintenance of vital physiological functions. These costs are expressed in joules per weight of soft tissue. For bivalves such as *P. canaliculus*, respiration represents investment in processes such as chemoreception, maintenance of ionic balance, selective filtration and rejection of unwanted particles, gape movements, avoidance behaviour, assimilation of calcium and shell accretion, waste removal, protein synthesis and defence from microbial pathogens. Combined, these costs can be expressed as the rate of oxygen consumption (ml O_2) per gram of tissue.

The experimental system comprised of nine respirometers supplied with seawater filtered to 0.5 μm from a bucket held in a temperature control unit maintained at 15°C (Figure 2.11 (b)). Respiration and excretion were measured simultaneously for eight individual mussels in eight of the 450 ml jacketed respirometers (Figure 2.11 (a)).

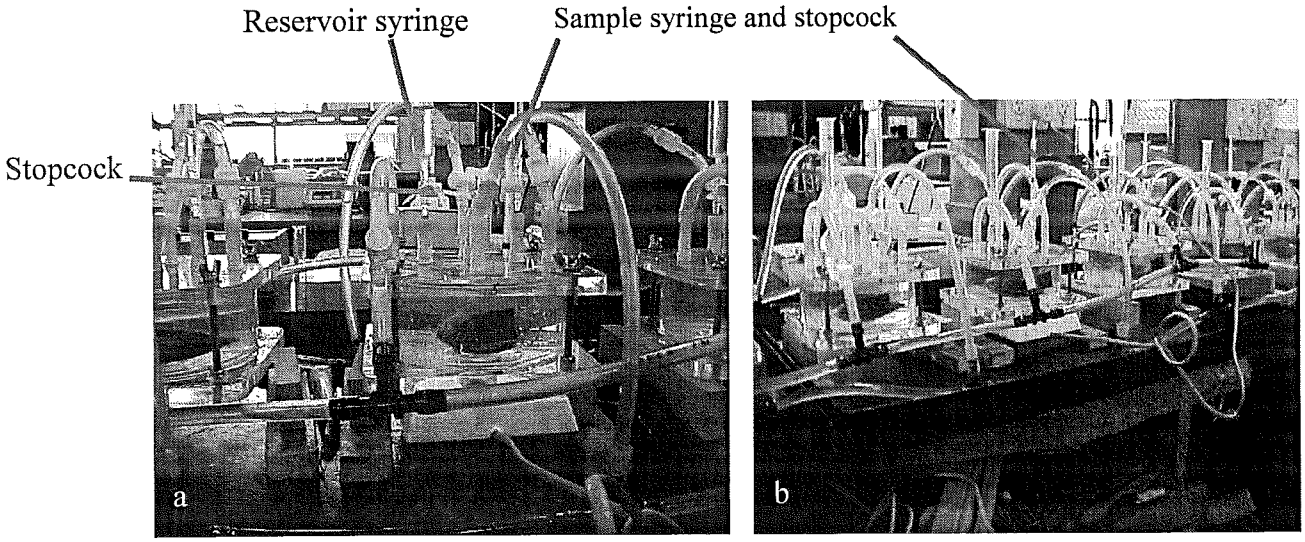


Figure 2.11: (a) Individual respirometer and (b) series of respirometers part of the nine respirometer system.

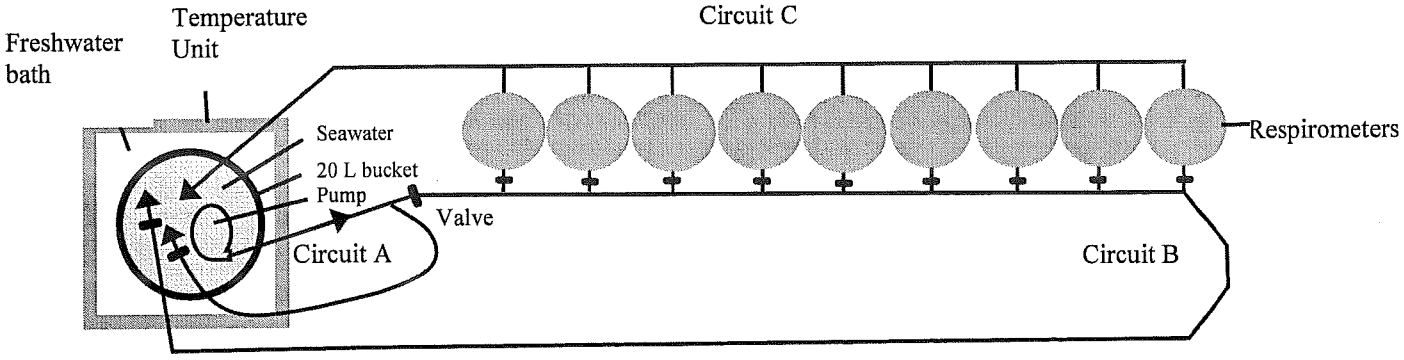


Figure 2.12: Diagram of seawater flow direction in three circuits between temperature control unit and nine respiromenters.

The ninth respirometer was used as a control and contained no mussel. The water transfer system for these respirometers consisted of three separate circuits for movement of seawater (Figure 2.12). These were installed to minimize bubbles in the respirometers. The delivery of bubble free seawater to respirometers was accomplished by first running seawater through circuit A for half hour at the start of the day's experimentation at the time

when the clearance rate experiment was being conducted. After this, water was run through circuit B for an hour and a half to remove air spaces in the supply line leading to individual respirometers. While this water circulation was being done, the valves to individual respirometers were closed so that no water entered the respirometers. After the clearance rates had been measured, mussels were transferred to the respirometers. Circuits A and B were closed and the valves for individual respirometers were opened and each respirometer in circuit C was placed on recirculation for two hours during which time mussels were not disturbed or fed. During this period of settling, freshwater at 15^{o}C was supplied to the temperature control water jackets surrounding each respirometry chamber. After this settling period, the inlet valve of each respirometer was closed for ten minutes and a 5 ml water sample was taken to determine the initial ammonium content of the filtered seawater. The inlet valve was opened again and the 5 ml seawater sample was replaced by new seawater. After this, the inlet and out valves for each respirometer were again closed. After ten minutes, a one ml seawater sample was taken for determination of the initial oxygen content of the water in each respirometer. An air driven magnetic stirring unit was placed under each respirometer fitted with a stirring bar so the water was continuously mixed for one hour. After one hour had elapsed, the final oxygen content of seawater in each respirometer was measured. This was done by withdrawing a one ml sample of seawater from the respirometers and injecting it into a Strathkelvin MC 100 microcell which housed a calibrated Strathkelvin model 1302 dissolved oxygen electrode. The partial pressure of oxygen in the water sample was read on a Strathkelvin oxygen meter Model 781.

Oxygen uptake by the mussels was assumed to be equal to the difference between the initial and final content of oxygen (μmoles O_2 L^{-1}) in the respirometer water.

This was calculated as:

$$\text{Respiration rate } (\mu\text{moles } O_2\ h^{-1}) = \frac{[O_2(t_0) - (O_2(t_1)] \times (V_r) \times 60}{(t_1 - t_0)}$$

where O_2 (t_0) = concentration of oxygen in the water (μmoles O_2 L^{-1}) at start of experiment

O_2 (t_1) = concentration of oxygen in the water (μmoles O_2 L^{-1}) at end of experiment

V_r = volume of respirometer minus the animal

t_0 = start time of the measurement period

t_1 = finish time of the measurement period

The concentration of oxygen in the seawater at time (t) was calculated as:

$$[O_2(t)] = \frac{[(\text{Experimental } PO_2 \text{ in mm Hg})]}{(PO_2 \text{ at air saturation})] \times 259.6 \text{ μmoles } O_2 L^{-1}}$$

where: Experimental PO_2 in mm Hg = Oxygen partial pressure at time (t)

PO_2 at air saturation = Oxygen partial pressure at air saturation

259.6 = Solubility of oxygen in seawater at 15°C

Excretion rate

Excretion rates also represent partitioning of a portion of the acquired energy into processes such as osmoregulation, maintenance during periods of low feed availability and waste removal. Excretion rate was determine using the phenol-hypochlorite method (Solorzano, 1969b).

$$\text{Excretion rate (μ moles } NH_4^+ \text{ hr}^{-1}) = \frac{[NH_4(t_1) - (NH_4(t_0)] \times (V_r) \times 60}{(t_1 - t_0)}$$

where: NH_4 (t_0) = concentration of ammonia in respirometer at time 0

NH_4 (t_1) = concentration of ammonia in respirometer at time 1

V_r = volume of respirometer minus the animal

t_1 = time at end of experiment

t_0= time at start of experiment

At the end of the experiment, before the mussels were dissected for determination of the dry weight, the volume of each mussel was determined by placing it in a filled cylinder made from PVC pipe and fitted with an overflow siphon. The amount of freshwater displaced by the mussel was siphoned off and weighed on an electronic balance. This weight was converted to volume and represented the volume of the mussel. The value was subtracted from the volume of water in the unoccupied respirometers and the corrected values used to calculate the total oxygen and ammonium concentrations in the seawater.

2.8 Scope for Growth (SfG)

The Scope for Growth (SfG) was calculated based on the equation:

$$SfG = C - (R + U)$$

where: C = Energy (J g^{-1} h^{-1}) acquired from feed particles cleared from the seawater

R = Energy (J g^{-1} h^{-1}) lost from respiration

U = Energy (J g^{-1} h^{-1}) lost from urine

2.9 Condition Index

After the clearance, respiration and excretion measurements had been determined, each mussel was weighed alive. The mussels were turned with their siphons facing downward to drain any free flowing water that may have been trapped inside the valves. After the live mussels were weighted, they were placed in a measuring cylinder filled with fresh water. The water which was displaced from the measuring cylinder by each mussel was then weighted. The weight of this water was converted to ml and used to represent the volume of each mussel. This volume was used to calculate the total volume of water used in the respirometry experiments.

After the mussels were removed from the measuring cylinder, they were then shucked and the tissue and shells were separated and placed into pre weighted aluminum foil boats (Figure 2.13). These were dried in an oven at 55°C for two days and reweighed to give the dry weight of the tissues and the shell (Figure 2.13). These were used to calculate the condition index (Crosby and Gale, 1990).

= tissue dry weight ^{x} 1000/(wet weight of live mussel – dry shell weight)

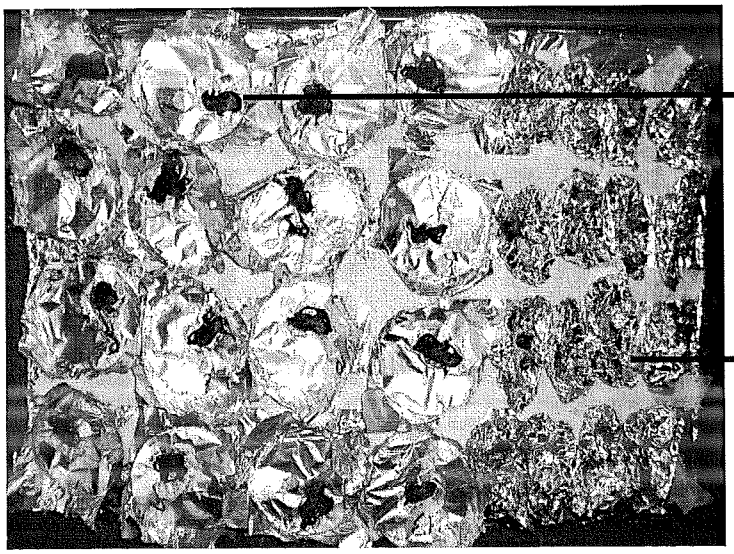


Figure 2.13: Sixteen samples of mussel tissues and shells after 48 hours drying in 55°C oven.

2.10 Statistical analysis and presentation

The data compiled were entered into Microsoft Excel^R spreadsheets and used to calculate clearance, respiration and excretion rates on a dry weight basis. The data for each experiment were pooled and tested for normality using the Shapiro-Wilk Test for normality in the Statistica^R Software Package (Tulsa, OK). If the distribution of the data was found to differ significantly ($p<0.05$) from the normal distribution, the data were ln transformed ($\ln 20 + x$). After the transformation, data for individual days were analysed for treatment differences using the single factor ANOVA option in the Data Analysis Add-in in Microsoft Excel^R. If significant differences were detected by the ANOVA, Tukey's honestly significant test (HSD) was performed *post hoc* using the Statistica^R software package to determine which treatments were different. After this, the entire data set was analyzed with repeated measures ANOVA using the Statistica^R Software. If differences were detected for any variable, Tukey'ss HSD test was used in *post hoc* evaluation of the significant differences detected. Results of the ANOVA and the repeated measures ANOVA were presented as "NS" if $p>0.05$, and ">0.001" if the p value determined was less than 0.001. Otherwise, significance levels (p) were presented as provided by the software.

The averages per treatment were presented graphically using Excel^R software. Error bars represented one standard error above and below the averages calculated. The averages on the graphs were offset horizontally to improve clarity of the representation.

Chapter 3 Factors which affect the respiration rate of *Perna canaliculus*

3.1 Introduction

Physiological and biochemical indices of mollusc fitness have been developed to understand how pollution affects energetic balance in aquatic species (Kiørboe *et al.*, 1985; Hawkins and Day, 1996). Indices such as mussel clearance (Toro *et al.*, 2003) and excretion rates (Elfwing and Tedengren, 2002a) have proven useful for assessment of the quality of waters impacted by urbanisation or industrial activities. Other indices such as respiration rate and oxygen to nitrogen (O:N) ratios have revealed few predictable patterns across different studies (Cheung and Cheung, 1995; Anandraj *et al.*, 2002; Mubiana and Blust, 2007). This may be because respiration rate in mussels is strongly affected by size, feeding rates, temperature, season and salinity (Bayne *et al.*, 1976a). This chapter describes six preliminary experiments designed to understand how certain factors affect the variability in experiments investigating the effects of trace metals on oxygen consumption rates in *Perna canaliculus*. These experiments were conducted because *P. canaliculus* aerobic metabolism can utilize over 20% of the energy in food for maintenance (Anderlini, 1992; Helson and Gardner, 2007). In order to use of SfG as an indicator of environmental stress, a convenient method of accurately measuring oxygen consumption for replicate samples of individual mussels was needed.

The term metabolism takes account of all the biochemical reactions necessary to utilize the potential energy and building blocks stored in food for maintenance and growth.

Metabolism is frequently reported as basal metabolic rate (BMR), which was first intensively studied and related to weight, height and age of humans (Harris and Benedict, 1919) and was used as a clinical tool to diagnose hypothyroidism (Hulbert and Else, 2004). BMR is the lowest level of metabolism capable of maintaining tissue and function in animals that are not under torpor or hibernation (da-Silva *et al.*, 2006). The BMR concept was used to develop the concept of standard metabolic rate (SMR) in order to compare metabolic rates across ectotherm species. BMR or SMR are measured directly by detecting the heat produced by metabolism using calorimetric studies. Alternatively, both BMR and SMR can be determined indirectly using oxygen consumption as an indicator for aerobic metabolic rate.

Oxygen consumption is an indirect method for quantifying the aerobic metabolic rate of bivalves which can be affected by factors such as size of specimens, temperature or availability of food which are highly variable in the natural environment. In addition, bivalve respiration rate may respond differently to the same type of stressors. For example, it has been shown that oxygen consumption may either increase, decrease or stay the same in bivalves exposed to different trace metals in the environment (Anandraj *et al.*, 2002). This may be because oxygen consumption rates are strongly influenced by intrinsic factors such as size and food intake as well as environmental factors such as temperature. Though respiration rates in molluscs have not proven to be reliable indicators of trace metal pollution, the linkage between chemicals in the environment, bivalve respiration rate and environmental modulators such as temperature have been appreciated for some time (Brand *et al.*, 1948). These relationships will continue to be important in the era of introductions of exotic bivalve species into new environments affected by climate change (Alexander and McMahon, 2004; Petes, 2007).

Previous studies have also shown that the rate of oxygen consumption in bivalves varies as a function of the soft tissue weight (Bayne *et al.*, 1976a), age of the mussels (Sukhotin and Portner, 2001), environmental conditions such as dissolved oxygen (Sobral and Widdows, 1997a) or carbon dioxide (Willson and Burnett, 2000) concentration of the holding water, salinity (Navarro and Gonzalez, 1998), temperature (Marsden and Weatherhead, 1998; Pilditch and Grant, 1999), physiological state of the mussels (Bayne *et al.*, 1976a), reproductive condition (Rueda and Smaal, 2004) and season (Huang and Newell, 2002)

etc. However, the main factors which affect respiration in bivalve mussels are size, level of feed, temperature, exposure to air, season and salinity (Bayne *et al.*, 1976a).

The first experiments were conducted in order to derive preliminary data on the rate of respiration in *P. canaliculus* and to check these rates against previous literature. A key objective was to identify factors which affect the value of oxygen consumption used to calculate SfG (Sections 1.10 & 2.8) of *P. canaliculus*. The goal was to eliminate the most important sources of variability which could affect the value determined for SfG in future experiments.

This chapter contains results from six experiments carried out between April 2005 and April 2006. These experiments investigated oxygen consumption rates of *P. canaliculus* mussels which were sourced from different locations on Banks Peninsula which were subject to different maintenance conditions at the University of Canterbury aquarium. This knowledge was essential to optimise the design of future experiments which would be used to identify the effects of copper, cadmium and salinity on the energetics of *P. canaliculus*.

3.2 Methods

List of experiments

Six experiments were conducted to determine factors which affect the respiration rates of mussels. These experiments were:

Experiment 1: Effects of two weeks storage of mussels on oxygen consumption.

Experiment 2: Effects of time of day on oxygen consumption in laboratory held and freshly collected and mussels.

Experiment 3: Oxygen consumption of farm and intertidal mussels.

Experiment 4: Effects of shell length and body mass of mussels on oxygen consumption.

Experiment 5: Oxygen consumption of fed and fasted mussels over 48 hours.

Experiment 6: Specific dynamic action of mussels.

Collection of mussels

The length of mussels used in these six experiments ranged between 5-8 cm. These were collected by inserting a sharp knife into the beds of juvenile and adult mussels and cutting the byssus threads to release clumps of fifteen or twenty mussels from the intertidal rock substrate at Taylor's Mistake. Mussels were then individually separated from the clumps with the knife and measured and counted in the field. The separated mussels were placed into buckets and immediately transported to the University of Canterbury aquarium where they were cleaned of barnacles and epibionts with a knife. Farm mussels were shipped by same day courier from an aquaculture farm in Pigeon Bay, Canterbury.

Maintenance prior to experiment

Mussels collected from the field were placed in 35 L plastic tanks fitted with an airstone, a standpipe and seawater supplied from a recirculation system managed by the SBS. These seawater systems were maintained at 13.5°C and 34 ppt salinity under artificial fluorescent light on a 12:12 hours on/off cycle. Mussels were held in these systems until respirometry was carried out. Mussels were generally not fed but, in the cases where feeding was done as a part of the experiment, the water supply to a tank was interrupted for two hours daily and fresh *Tetraselmis chuii* applied to the tank.

When respirometry experiments were to be conducted, each mussel's byssal threads were severed with a knife the day before and the mussels removed from the aquarium tanks. These mussels were placed in the respirometer chambers on the evening of the day before the respirometry procedures were to be carried out. The respirometry system was then placed on recirculation overnight before oxygen consumption exercises were conducted on the subsequent morning.

Respirometers and oxygen determination

For experiments one, two and four, the respirometers used were four opaque grey colour 713 ml polyvinyl chloride (PVC) respirometers fitted with transparent covers and plastic pottle spacers to achieve a volume of 503 ml. Each respirometer was individually supplied with seawater via a 50 L h^{-1} pump. Experiments three, five and six were conducted in nine transparent perspex respirometers (Figure 3.1) arranged in parallel (Figure 3.2) and attached to a supply of seawater using a submersible 500 L hr^{-1} pump (Figure 2.12.).

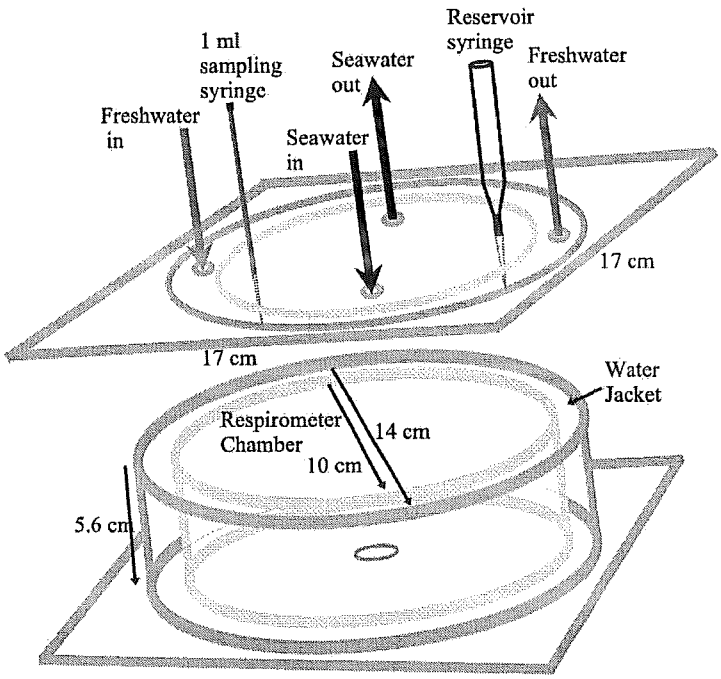


Figure 3.1: View of water inlets and outlets on individual respirometers.

The diameter of the respirometers used in experiments three, five and six was 14 cm and the height was 5.6 cm. Each contained an average of 445 ml of water space and each was fitted with one inlet and one outlet valve for seawater. In addition to valves, each outlet contained a check valve to prevent backflow of seawater from the outlets of respirometers furthest away from the supply pump into the outlets of respirometers closer to the supply pumps (Figure 3.2).

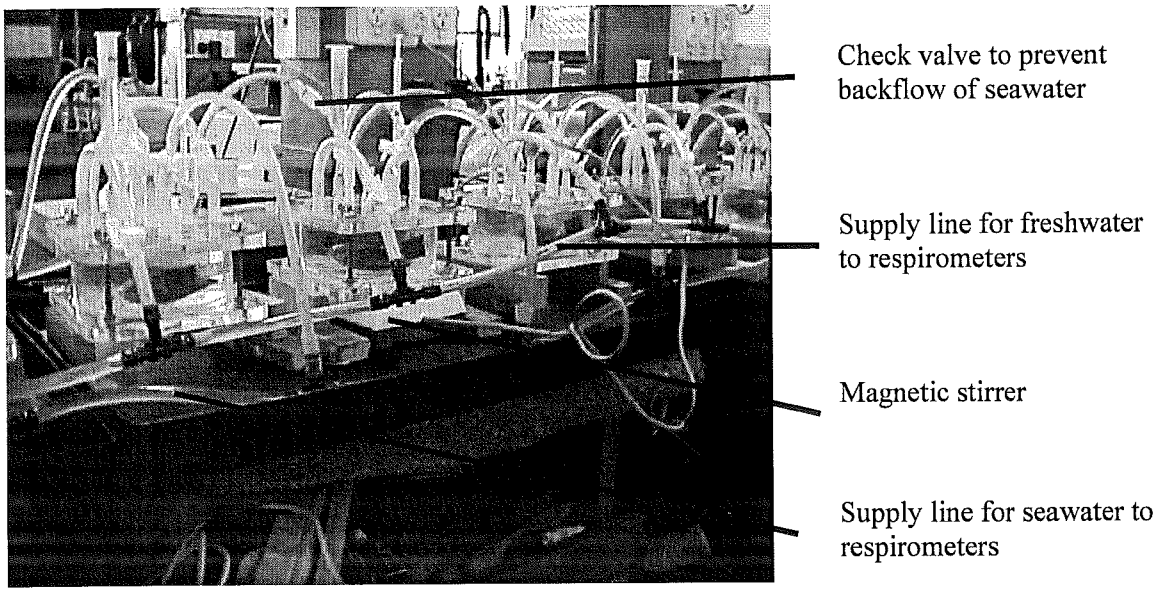


Figure 3.2: Freshwater and seawater supply lines to four of nine respirometers in parallel.

This arrangement was to ensure that when respirometers were under recirculation overnight, no respirometer could become a dead spot where metabolites would accumulate. The fifth respirometer which was a control contained no mussel and was used to measure background respiration in the seawater. This control respirometer was used to calculate oxygen consumption due to biological or biochemical oxygen demand (BOD) in the seawater used in the experiments.

In order to decrease the value of the biological oxygen demand in the controls, seawater used in these experiments was filtered with a Doulton ceramic water filter candle (Staffs, England) which removed 99.9% of biological materials between size 0.5-0.8 μm. This size range ensured that most algae and bacteria including *E. coli*, *Vibrio* and *Salmonella* would be eliminated from water used in the respirometry experiments. Each of the respirometry chambers used in experiments three, five and six had a water jacket containing freshwater maintained at 15°C (Figure 3.3). The water jacket was supplied with freshwater from a chilled water-bath fitted with a temperature controlled water heater set at 15°C.

The freshwater in this system was maintained at 15°C with an element heater placed inside a refrigerated water bath (Figure 3.3). Freshwater was pumped from the temperature controlled water bath into all the water jackets using one submersible 500 L hr^{-1} electric pump. In this way, water was circulated continuously among the water jackets of all the respirometers and the temperature controlled water bath (Figure 3.3). When respirometry experiments were conducted, provisions were also made to reduce errors related to inadvertent stress in the mussels. Because it has previously been shown that bivalves may suspend filtering activity because of movement and vibrations in ordinary laboratory settings (Kim *et al.*, 1999), mussels in these first experiments were placed in respirometers overnight and filtered seawater was recirculated into the chambers. This was done in order to acclimate the mussels to a lack of a natural substrate for byssal attachment, low seston in the seawater and confinement to an unnaturally small space. During this settling period, no algal feed was provided to the mussels. Just before the oxygen consumption experiments were carried out, all bubbles which had accumulated in the respirometers overnight were removed and all respirometers were closed within one minute of each other. Ten minutes were allowed to elapse before the first water sample was taken to determine the initial oxygen content of the water. This was done by opening the stopcocks (Figure 2.11 (a & b)) of the sampling and the reservoir syringes and removing water samples from the respirometers using a 1 ml plastic syringe. After this, the stopcocks of the sampling and reservoir syringes were immediately closed. The 1 ml of sample was then injected into a Strathkelvin MC 100 microcell fitted with a Strathkelvin model 1302 dissolved oxygen electrode and read on a Strathkelvin oxygen meter Model 781. The dissolved oxygen was read on the meter after ninety seconds. Water surrounding the oxygen electrode of the microcell was maintained at 15°C using water supplied from the temperature controlled water bath (Fig 3.3).

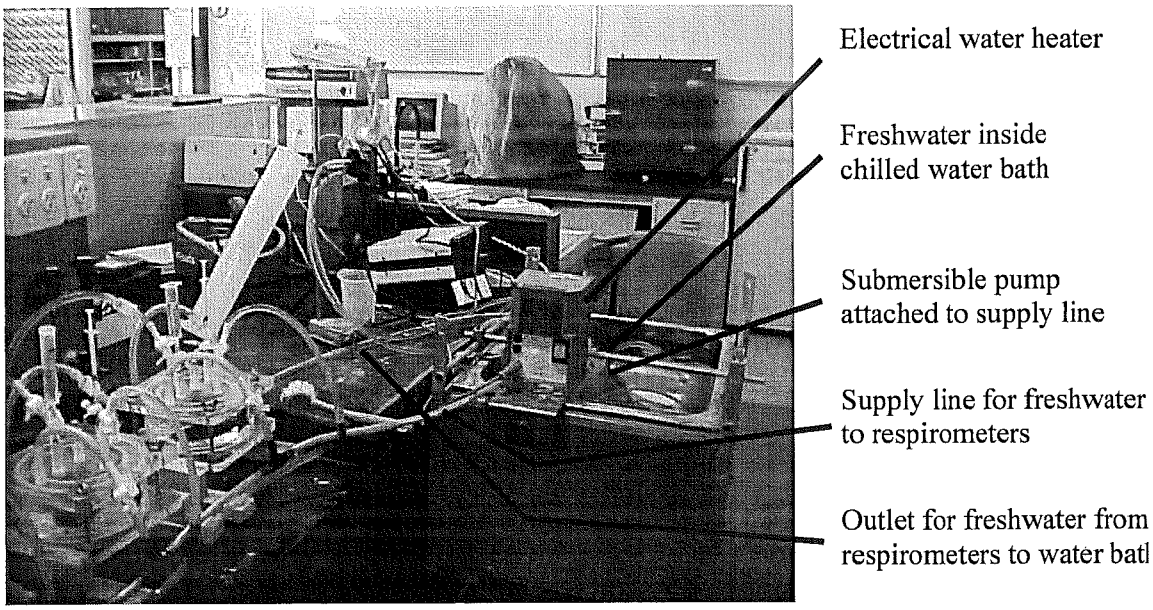


Figure 3.3: Chilled water bath with heated temperature control unit used to supply water maintain water at 15°C

Experimental design

Experiment 1: Effects of two weeks storage of mussels on oxygen consumption (31 August, 2005).

Respiration rates was determined on 31 August, 2005 on n = 2 intertidal mussels which had been collected on 29 August, 2005 and stored for two days and on n = 2 intertidal mussels which had been collected on August 18, 2005 and maintained without feeding at the SBS aquarium. The mussels selected were 4 $\pm$ 0.5 cm in length. Oxygen consumption per mussel was calculated by recording the rate of oxygen depletion in 503 ml of 34 ppt salinity seawater maintained at 15°C.

The respiration rates of individual mussels were determined twice for each animal over two one hour periods. During each one hour period, measurements of the declining oxygen concentration in the respirometer water were conducted at intervals of 20 minutes. The two one hour periods were conducted in succession on the same day for each mussel. At

the end of these procedures, mussels were shucked and the dry weights of soft tissue and shells determined. Respiration rates were then calculated and expressed on a dry weight basis.

Experiment 2: Effects of time of day on oxygen consumption in laboratory held and freshly collected and mussels (2 September, 2005).

Mussels belonging to the same sets of recently collected and laboratory held mussels used in experiment 1 were used to determine the rates of respiration at three different times during the day. The recently collected mussels had been stored in the aquarium for two days. The laboratory held animals had been kept at the SBS aquarium for 15 days and maintained on a 12:12 hours light/dark cycle using artificial lighting. In this experiment, respiration rates of n = 2 recently collected and n = 2 previously collected mussels were measured at 20 minutes over one hour starting at 11:00 AM, 2:00 PM and 6:00 PM. As in the case of experiment one, mussels of 4 cm shell length were selected.

Experiment 3: Oxygen consumption of farm and intertidal mussels (July 15, 2006).

For this experiment, *Perna canaliculus* mussels of various sizes were collected on July 7, 2006 from Taylor's Mistake, Christchurch and transferred to the University of Canterbury fourth floor aquarium. Four mussels of similar shell length (7.5 cm) were selected from these mussels for this experiment. Four mussels in the same size class were also selected from mussels which had been recently received from an aquaculture facility at Pigeon Bay. The oxygen consumption using jacketed temperature controlled respirometers was determine for the n = 4 intertidal and n = 4 farm mussels at intervals of four hours over a period of 28 hours. One respirometer which contained only seawater was used to determine the background oxygen consumption of the seawater. This was subtracted from the value of total oxygen consumed in each respirometer.

On the evening of the day before respirometry was carried out, the byssal threads of eight mussels in the aquarium were severed. The mussels were collected and placed in the eight respirometers overnight. These mussels were left to acclimate to 15°C by circulating seawater maintained at this temperature between the respirometers and a 20 L bucket in a

waterbath (Figure 2.12). Temperature in the 20 L bucket was maintained by a water bath maintained at 15 °C.

At 8:00 AM on the day of the experiment, circulation to the respirometers was suspended and a portion of the seawater in each respirometer was removed so that all bubbles that had accumulated from the overnight water circulation could be eliminated. When all bubbles were removed from each respirometer, a brief period of recirculation with new seawater was carried out. After this recirculation with fresh seawater, the respirometers were closed and the oxygen consumption over one hour was measured in the nine respirometers. This was done by sampling 1 ml of seawater at the beginning and the end of a 60 minute period.

After measuring the oxygen consumption over one hour, the respirometers were once again placed on recirculation for about 2.5 hours before oxygen consumption was again determined four hours after the previous reading. At the end of 28 continuous hours of oxygen consumption experiments, mussels were removed from the respirometers and briefly held with the valve opening facing downward to drain all free water out of the valves. Mussels were then weighed wet then shucked and the wet weights of the shell and viscera taken. The wet shells and viscera were then dried for 6 days at 55°C as in Section 2.9.

Respiration rate for each mussel was determined by calculating difference in oxygen content of the seawater in the respirometers before and after each 60 minute oxygen consumption interval. The respiration rates of four recently collected intertidal and farm mussels were then compared with repeated measures ANOVA.

The condition indices (CI) of the mussels were calculated based on the dry weights of the viscera and shell and the wet weight of the live mussel as in Section 2.9:

$$CI = \text{dry soft tissue wt (g)} \times 1000 / \text{internal shell cavity capacity}$$

Where CI = condition index,

Internal shell cavity capacity = total whole live weight (g), in air, of a cleaned mussel - dry shell weight.

The condition indices and dry weights of the four farm and four intertidal mussels were then compared with two sample Student-t tests.

Experiment 4: Effects of shell length and body mass of mussels on oxygen consumption (July 16, 2006).

This evaluation comprised two oxygen consumption experiments. In the first experiment, respiration rates of two small and two large mussels which had been collected from Taylors Mistake on July 7 were compared using 713 ml respirometers fitted with spacers to provide a working volume of 503 ml of seawater. This experiment was also used to develop a mathematical relationship between the dry weights of the mussels and the rate at which these animals consumed oxygen. In the second set of oxygen consumption experiments, respiration rate of three small, three medium size and two large mussels were compared at four hour intervals between 8:00 AM and 8:00 PM. Data from both experiments were compiled and used to investigate the relationship between oxygen consumption and the mass of the mussels.

Experiment 5: Oxygen consumption of fed and fasted mussels over 48 hours (June 8, 2006).

Mussels used in this experiment were cut from the intertidal rock areas at low tide on the 4 th and 13th April 2006 at a location south of the bay at Taylors Mistake. Mussels were transported in plastic buckets to the SBS. At the SBS, mussels were brush cleaned before measures of the shell length and width were taken using a digital calliper and the wet live weight measured with a digital balance. Mussels were then attached by their shell to 3 x 3 cm perspex plates using cyanoacrylate glue. The plates were then suspended from a plastic rod placed across the top of two separate experimental tanks. Mussels in tank one were fed every other day with fresh *Tetraselmis chuii* and *Isochrysis galbana*. Mussels in tank 2 were never given algal feed.

After 56 days, four mussels from each tank were removed and individually placed in a respirometer chamber. Each mussels was held in an upright position by suspending the attachment perspex plate from the bottom surface of the lid of each respirometer using small pieces of velcro. In this way, fed and unfed mussels were placed alternatively in each respirometer, leaving the fifth respirometer without a mussel. All respirometers were then placed on recirculation overnight using the parallel closed loop system described in Section 2.7. On the following morning, when oxygen consumption procedures were started, recirculation was suspended and a portion of the water in each respirometer was

removed along with the bubbles that had accumulated. Respirometers were then refilled and closed. The oxygen consumption for each mussel was then determined over a one hour period. After the final oxygen consumption was determined for the mussel in the last respirometer, new filtered seawater was introduced into the respirometer system. The system was then placed on recirculation for 2.5 hours until the next sampling period. This process of respirometry, renewal of the seawater, recirculation then respirometry was repeated 12 times over a period of 48 hours. After the last oxygen consumption measurement, the mussels were shucked and tissues separated from the shells for drying.

Experiment 6: Specific dynamic action of mussels (August 7, 2006).

Eight mussels which were collected from Taylor's Mistake on July 25 and held at the SBS aquarium were removed from the aquarium tanks on August 7. These mussels were prepared for the current experiment designed to determine the duration of the specific dynamic action of *Perna canaliculus* by settling them in undisturbed respirometers overnight under recirculation at 15°C. Each respirometer was part of a system recirculating filtered seawater between the respirometers and a 20 L bucket held at 15°C in a chilled water bath (Section 2.7).

At 8:00 am on the day of the experiment, recirculation was suspended and ¼ of the water in each respirometer was removed with a 50 ml syringe in order to remove bubbles which had accumulated from overnight recirculation of the system. Respirometers were then refilled with fresh seawater and placed on recirculation briefly. After this, oxygen consumption determinations were carried out for all 9 respirometers for a one hour period. After this period, water recirculation was stopped for the entire system and the respirometers opened and the mussels removed. All eight mussels were then placed for 1 hour in 4 L buckets containing 50 ml of fresh *Tetraselmis chuii*. Mussels were then returned to the respirometers after 1 hour of feeding and the respirometers closed and placed on recirculation for 30 minutes. Respirometers where then opened and bubbles removed. After this, the respirometers were refilled and oxygen consumption determined at 1 hour after feeding. After this, respirometers were place on recirculation for 30 minutes then they were closed to determine respiration rates at 2, 3, 4 and 5 hours after feeding. Following these determinations, the respirometers were kept closed and placed on

recirculation for 19 hours. Oxygen consumption was then determined at 24 hours after feeding. At the end of the oxygen consumption experiments, the soft tissue and shells were separated and dried over three days to determine the dry weights of the soft tissues and shells.

3.3 Results

Experiment 1: Effects of two weeks storage on oxygen consumption.

The average oxygen consumption rates for the recently harvested mussels and the mussels held in the SBS aquarium were 0.222 ± 0.013 ml O_2 $g^{-1}h^{-1}$ and 0.098 ± 0.009 ml O_2 $g^{-1}h^{-1}$ respectively.

Table 3.1: Mean dry weight and oxygen consumption rates of recently collected intertidal and laboratory maintained *P. canaliculus* mussels.

Source of mussel	Dry weight (g)	Oxygen consumption (ml O_2 $g^{-1}h^{-1}$)
Recently harvested	0.839	0.222
Laboratory held	0.790	0.098

The mean dry weights of the two recently harvested and laboratory maintained mussels were 0.839 g and 0.790 g, which were statistically similar ($t_{(1,2)}$ = 0.35, p = 0.756). Therefore, the differences detected in oxygen consumption were likely the result of variations in metabolic rate of the mussels and not as a result of differences in the tissue weight.

Experiment 2: Effects of time of day on oxygen consumption in laboratory held and freshly collected and mussels.

When the respiration rates of laboratory held and recently harvested mussels were compared, two trends were noted. Firstly, there was an apparent difference in respiration rates between recently collected mussels and the laboratory held mussels (Figure 3.4). Secondly, respiration in both sets of mussels appeared to be declining over time.

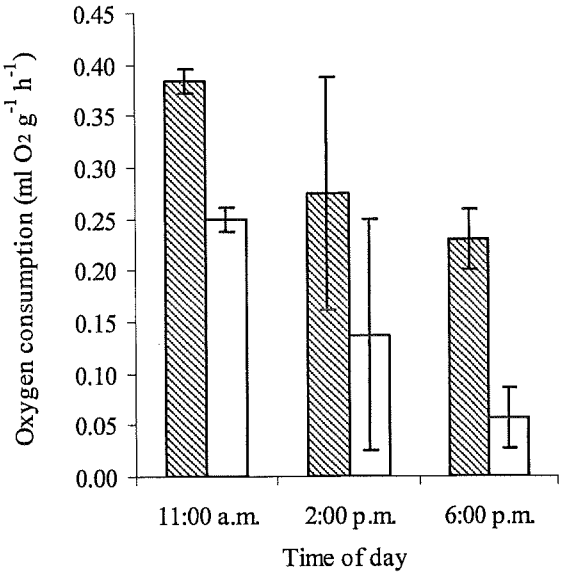


Figure 3.4: Oxygen consumption (ml O_2 $g^{-1}h^{-1}$) +/- SE of n = 2 recently harvested (lines) and n = 2 laboratory (white) held *Perna canaliculus* at 11:00 AM, 2:00PM and 6:00 PM.

Although differences in respiration between the two treatments were apparent in the mean values at 11:00 AM and 6:00 PM, ANOVA of data for individual days showed that differences in respiration were significant only at 6:00 pm (ANOVA $F_{(1,3)} = 34.11$, $p = 0.03$).

Repeated measures ANOVA (Table 3.2) performed on the data for the three sampling periods showed that over the seven hour course of the experiment, the average oxygen consumption rate for the recently harvested mussels (0.30 ± 0.05 ml O_2 $g^{-1}h^{-1}$) was not significantly different from the 0.15 ± 0.06 ml O_2 $g^{-1}h^{-1}$ recorded for the laboratory held mussels (Table 3.2).

Table 3.2: Repeated measures ANOVA of effects of time of collection and time of respirometry on oxygen consumption of recently collected and laboratory held *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Time of collection	<0.001	1	<0.001	4.206	NS
Time of respirometry	<0.001	2	<0.001	5.515	NS
Time of collection*Time of respirometry	<0.001	2	<0.001	0.092	NS

Repeated measures ANOVA also showed that the effects of time of day at which respirometry was carried out was not significant. The mean dry weight of recently harvested mussels (0.726 $\pm$ 0.04 g) and the laboratory held mussels (0.710 $\pm$ 0.02 g) were also statistically similar ($t_{(1,2)} = 2.93$, $p = 0.727$).

Experiment 3: Oxygen consumption of farm and intertidal mussels.

Over of the 28 hours of respirometry, oxygen consumption was higher for intertidal mussels compared to the farm mussels at all of the time intervals (Figure 3.5).

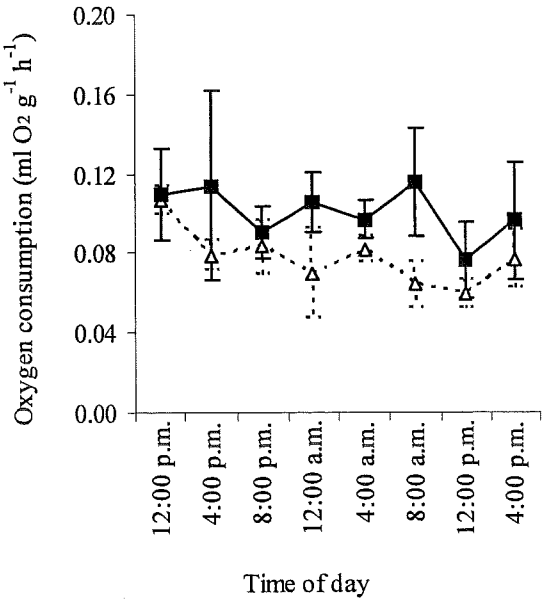


Figure 3.5: Changes in respiration rate (ml O_2 $g^{-1}h^{-1}$) +/- SE of 4 farm (△) and 4 intertidal (□) mussels over 28 hours.

After the first sampling when respiration rates for the two types of mussels were virtually identical, the rates for the farm mussels declined from the initial value of 0.106 ± 0.007 ml O_2 $g^{-1}h^{-1}$ to a low value of 0.08 ± 0.007 ml O_2 $g^{-1}h^{-1}$. Mean consumption rates by intertidal mussels remained stable with values near to the initial respiration of 0.110 ± 0.023 ml O_2 g^{-1} h^{-1} for the duration of the 28 hours of the experiment. Analysis of the data using repeated measures ANOVA showed that the mean rate of 0.077 ± 0.01 ml O_2 g^{-1} h^{-1} for the farm mussels was statistically similar to the 0.101 ± 0.02 ml O_2 $g^{-1}h^{-1}$ for the intertidal mussels over the duration of the experiment (Table 3.3). Also, the effects of time of day and interaction between source of mussels and time of day indicated that no differences could be attributed to either of these sources of variation (Table 3.3). This implies that no diurnal respiratory patterns could be detected.

Table 3.3: Repeated measures ANOVA of respiration rates of farm and intertidal mussels.

Source of Variation	SS	df	MS	F	p
Source of mussels	<0.001	1	<0.001	3	NS
Time of day	<0.001	7	<0.001	1	NS
Source of mussels*Time of day	<0.001	7	<0.001	0	NS

The condition index and dry weights of farmed mussels were considerably greater than the condition and weight of intertidal mussels (Fig 3.6(a) and 3.6(b)).

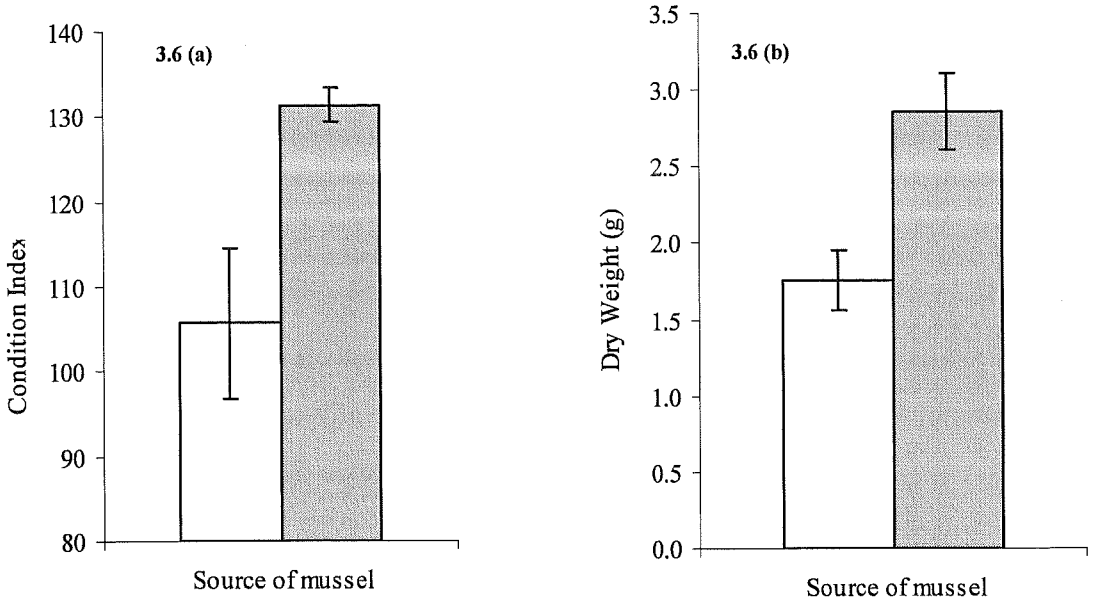


Figure 3.6: Condition indices +/- SE and dry weights ± SE of 4 intertidal (white) and 4 farm (diagonal lines) mussels.

Comparison of the condition of the two sets of mussels utilizing a Student-t test showed that farm mussels had significantly greater condition ($t_{(1,6)}$ = -2.83, p = 0.030). This difference in CI was primarily because the tissue dry weights of the farm mussels were significantly greater ($t_{(1,6)}$ = -3.49, p = 0.013) than the weights of the intertidal mussels.

Experiment 4: Effects of shell length and body mass of mussels on oxygen consumption.

Results of experiment four which determined the respiration rates of different size mussels over 60 minutes showed that respiration rate and dry weight did not vary linearly. The dry weight of mussels in the first experiment ranged between 0.666 g and 5.518 g. The relationship between dry weight and respiration rate was best described by a power equation where respiration rate (y) = 0.3261 (dry weight)$^{0.5365}$. In the case of the second experiment, respiration rate measured over sixty minutes was described by equation (y) = 0.1731 (dry weight) $^{0.3656}$ with mussel dry weights ranging between 0.241 and 2.238 g. The r^2 for the first set of oxygen consumption determinations was 0.8736 but this value was 0.638 for the second experiment which utilized a smaller range of mussel dry weights. Combining data from both experiments, the respiration rate for the pooled data was related

to dry weight by the equation (y) = 0.2352 (dry weight) $^{0.5766}$ with r^2 for the combined data being 0.7695 (Figure 3.7).

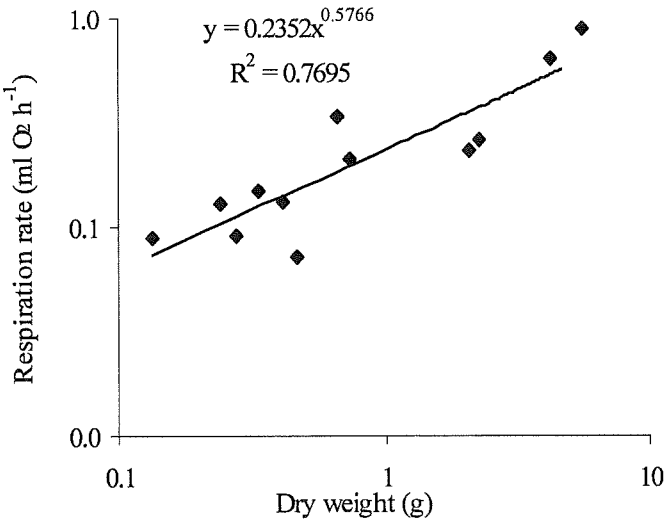


Figure 3.7: Respiration rate (ml O_2 h^{-1}) +/- SE of *Perna canaliculus* as a function of dry weight of tissue.

Analysis of the respiration rates of small, medium and large mussels over the twelve hours of measurements showed that there were no differences in respiration detectable between animals of different sizes (Fig 3.8, Table 3.4). Small mussels averaging 0.219 g dry weight displayed average respiration of 0.217 $\pm$ 0.068 ml O_2 g^{-1} h^{-1} while medium sized mussels of 0.406 $\pm$ 0.061 g dry weight recorded oxygen consumption at a rate of 0.156 ml O_2 g^{-1} h^{-1} and large mussels of 2.146 g dry weight respired at 0.090 $\pm$ 0.005 ml O_2 g^{-1} h^{-1}. Although there were no significant effects of tissue dry weight on respiration rate, a significant effect of time of day on respiration rate of the mussels was noted (Table 3.4). This effect of time was a result of a decline in respiration from 0.279 ml O_2 g^{-1} h^{-1} at 8:00 AM to 0.106 ml O_2 g^{-1} h^{-1} at 8:00 PM.

Table 3.4: Repeated measures ANOVA of small, medium and large mussels over 12 hours

Source of Variation	SS	df	MS	F	P-value
Size	0.079	2	0.039	2.045	NS
Time of respirometry	0.18	3	0.06	5.305	0.011
Time of respirometry*Size	0.031	6	0.005	0.455	NS

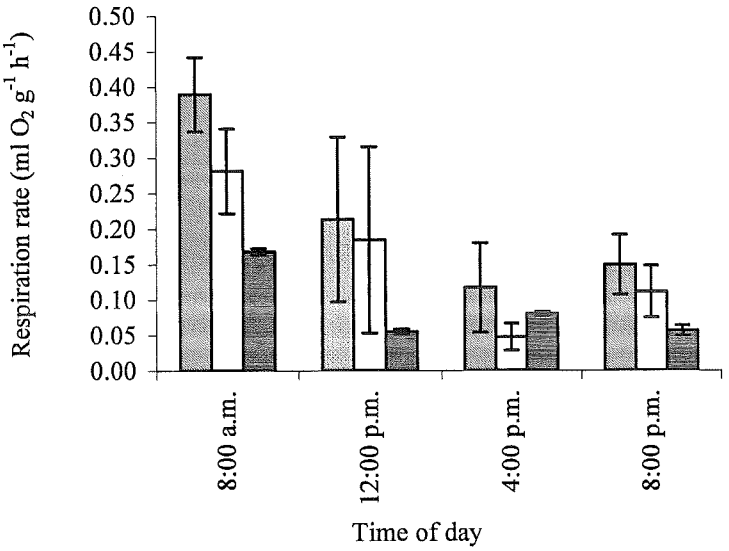


Figure 3.8: Changes in respiration rate (ml O_2 $g^{-1}h^{-1}$) +/- SE of small (diagonal lines), medium (white) and large (horizontal lines) mussels over 12 hours.

Experiment 5: Oxygen consumption of fed and fasted mussels over 48 hours.

Respiration rates of the four unfed and four fed mussels determined over the 48 hours were variable but showed that fed mussels were metabolically more active (Fig 3.9). The average rate of oxygen consumption for the fed mussels was 0.21 $\pm$ 0.08ml O_2 g^{-1} h^{-1} whilst 0.097 $\pm$ 0.05 ml O_2 g^{-1} h^{-1} was the rate recorded for the unfed mussels. At the first reading, fed mussels consumed 0.384 $\pm$ 0.05ml O_2 g^{-1} h^{-1} versus the 0.119 $\pm$ 0.06 ml O_2 g^{-1} h^{-1} consumed by unfed mussels. These distinct metabolic rates (ANOVA $F_{(1,7)} = 10.51$, $p = 0.018$) detected at the first sampling were not detected at the second sampling because of a decline in the rate recorded for fed mussels to 0.218 $\pm$ 0.1 ml O_2 g^{-1} h^{-1}. Despite this decline, respiration remained reasonably high for the fed mussels over the first 20 hours. This was followed by a declined to near zero values for an eight hour period. These near

zero values were recorded at 5:00-9:00 PM on the second day of measurements. The data for these points were therefore not included in the data analysis. During this period, the respiration of unfed mussels was also low but was consistent with previous readings.

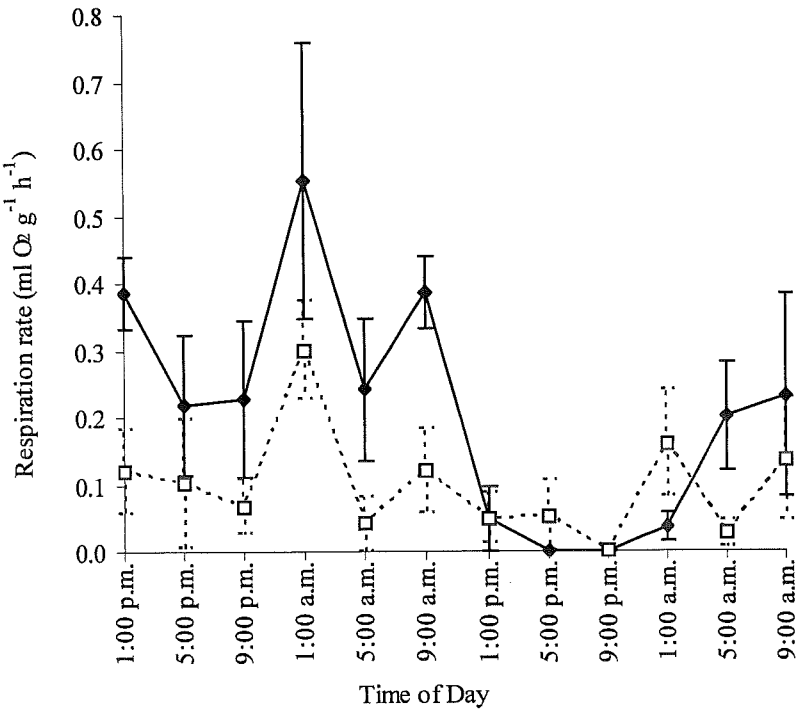


Figure 3.9: Changes in respiration rate (ml O_2 $g^{-1}h^{-1}$) +/- SE of 4 fed (◇) and 4 unfed (□) *Perna canaliculus* over 48 hours.

Table 3.5: Repeated measures ANOVA of fed and unfed mussels over 12 hours.

Source of Variation	SS	df	MS	F	P-value
Feeding status	0.001	1	0.001	15.23	0.008
Time	0.002	10	0	3.33	0.002
Feeding status*Time	0.001	10	0	1.17	NS

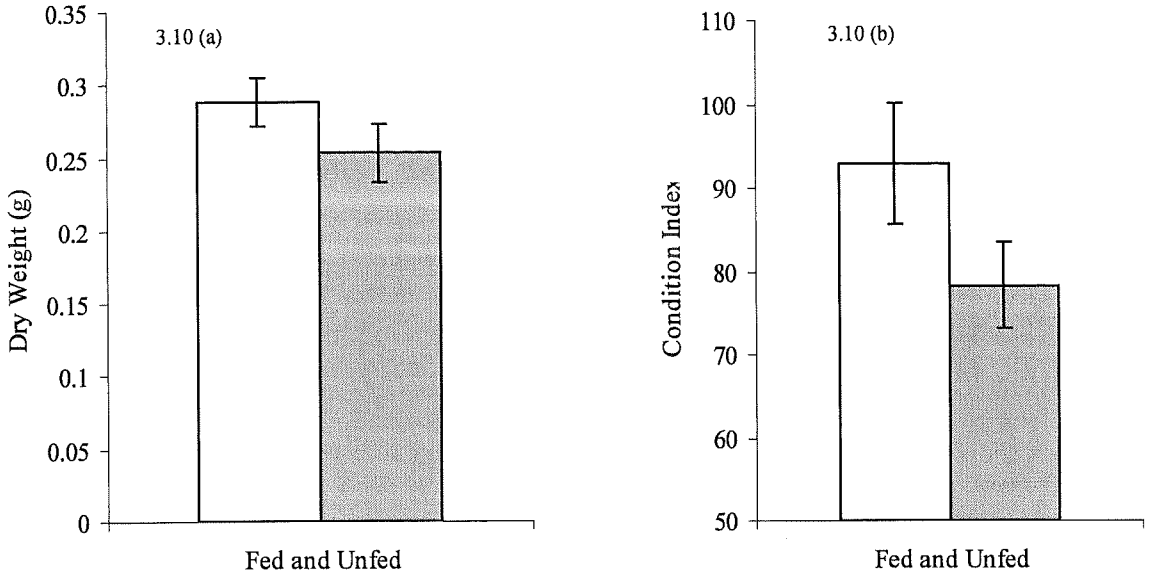


Figure 3.10: Condition indices +/- SE and dry weights +/- SE of 4 fed (white) and 4 unfed (diagonal lines) mussels.

The lack of significant differences in respiration rates between the fed and unfed which was detected at the second sampling in the experiment continued for ten of the eleven data points between hours four and forty eight of the experiment. These were analysed with repeated measures ANOVA using the application of feed as one variable and the time of respirometry as the other. The mean respiration rates were 0.097 $\pm$ 0.05 ml O_2 g^{-1} h^{-1} for unfed mussels and 0.210 $\pm$ 0.08ml O_2 g^{-1} h^{-1} for fed mussels were significantly different over the 48 hour duration of the experiment (Table 3.5). Examination of the differences with Tukeys HSD showed that respiration at 1:00 AM on the first day was significantly higher than respiration at 5:00 PM, 9:00PM, 1:00 AM and 5:00 AM on the second day of the experiment.

The differences over time may also be due to a marked decline in respiration between 1 pm and 9 pm on the two days. This decline was much greater on the first day. On the second day, the zero respiration was recorded for mussels at 5:00 PM and 9:00 PM. These results show that after a period of two months, mussels that were fed and unfed showed different respiration patterns. The respiration in unfed mussels were low but stable over the forty eight hour period. The rate of the fed mussels were high for the first day but declined to near zero values after twenty-four hours of continuous monitoring.

The condition indices determined for the four unfed and fed mussels were 78.30 and 92.88 respectively and were statistically similar (ANOVA $F_{(1,7)} = 2.74$, $p = 0.15$). The dry weights of 0.288 g and 0.253 g for fed and unfed mussels were also statistically similar (ANOVA $F_{(1,7)} = 1.74$, $p = 0.24$).

Experiment 6: Specific dynamic action of mussels.

The respiration rates recorded before the mussels were fed were the lowest for the experiment (Figure 3.11).

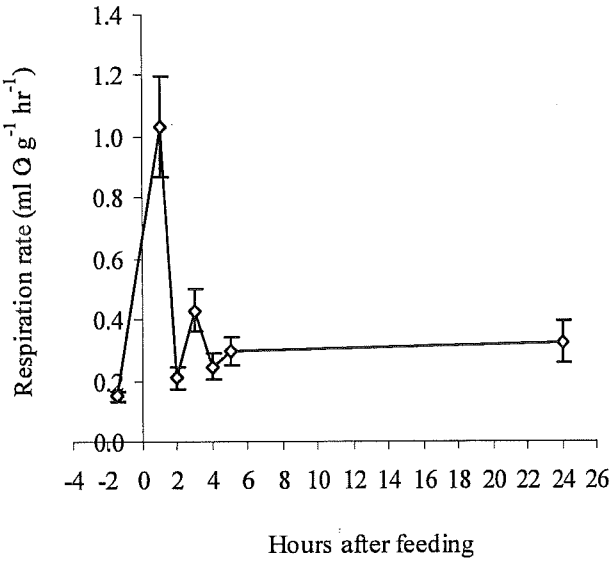


Figure 3.11: Respiration (ml O_2 g^{-1} h^{-1}) +/- SE of 8 mussels 1.5 hrs before feeding and at hourly intervals after feeding.

Respiration increased immediately after feeding and were highest at one hour post-feeding. After this, respiration returned to pre feeding levels. Analysis of the data showed that significant differences in respiration rates were recorded among the seven sampling times (ANOVA $F_{(1,6)} = 13.58$, $p<0.001$). This was due to the substantial rise in respiration at one hour after feeding. The differences between the average of 0.15 ml O_2 g^{-1} h^{-1} before feeding and the averages of 0.21 ml O_2 g^{-1} h^{-1}, 0.43 ml O_2 g^{-1} h^{-1}, 0.24 ml O_2 g^{-1} h^{-1} and 0.30 ml O_2 g^{-1} h^{-1} from two to and five hours after feeding were not significant. This indicated that the SDA only lasted for a brief period of less than two hours.

3.4 Discussion

The measurement of metabolic rates has allowed researchers to determine how various environmental variables affect growth, health, reproduction and survival of bivalves (Bayne *et al.*, 1976a; Hummel *et al.*, 2000; Martınez *et al.*, 2000). In more complex studies, the mechanisms of how the metabolic rates change in response to variables such as level of food have provided insight into the linkage between the environmental and intrinsic factors such as feed absorption efficiencies which can affect population structures (Gardner, 2000). The results in this chapter showed that changes in the metabolic rate of *Perna canaliculus* reflected the conditions of the aquatic environment which these mussels occupied. Specifically, the experiments in this chapter showed that laboratory maintenance, food and storage time need to be taken account of when conducting research on respiration of *P. canaliculus*.

Laboratory maintenance

Effects of two weeks storage

The first experiment conducted in this chapter addressed the question of whether the metabolism of *Perna canaliculus* mussels is affected by medium or long term storage in the SBS recirculating seawater facility. The first experiment compared the metabolic rates of mussels which had been collected from field sites the previous day with mussels held in the SBS facility for two weeks. Because the recently collected mussels were not fed during or before these experiments, it is likely that these were not in the process of digesting a significant amount of feed. The respiration rate of 0.222 ml O_2 $g^{-1}h^{-1}$ determined for the recently collected mussels in the current research was lower than 0.39 ml O_2 $g^{-1}h^{-1}$ (Marsden and Shumway, 1993) and 0.45 ml O_2 $g^{-1}h^{-1}$ (Gardner and Thompson, 2001) recorded for *P. canaliculus* previously but was close to 0.256 ml O_2 $g^{-1}h^{-1}$ (Helson and Gardner, 2007) documented for this species.

The rate of 0.098 ml O_2 $g^{-1}h^{-1}$ determined for the laboratory held mussels in the current research shows that within two weeks of holding mussels under laboratory conditions, there was a decline in the respiration. A significant difference was not detected in the second experiment where the mean respiration rate for the field mussels was 0.30 ± 05 ml O_2 $g^{-1}h^{-1}$ compared to the 0.15 ± 0.06 ml O_2 $g^{-1}h^{-1}$ for laboratory held mussels. This result

may have been related to the small sample size and the decline in respiration by both treatments over the seven hour period. Such a decline in respiration has been recorded for freshwater snails (Brand *et al.*, 1948) held under laboratory conditions for seven weeks. In one early study, a decline of over fifty percent of the initial oxygen consumption (expressed per mussel) from 0.40 ml O_2 $snail^{-1}$ h^{-1} to 0.15 ml O_2 $snail^{-1}$ h^{-1} was noted for *Astraloribis glabratus*. Similar decreases were noted for *Helisoma duryi*, *Physa gyrina* and *Physa* mussels after one week (Brand *et al.*, 1948).

Results from the present study also agree with early work on marine bivalves which showed a decline in respiration in *Mytilus edulis* which were fed with unialgal cultures of *Tetraselmis suecica* (Bayne, 1973b). Also, recent research has shown that the respiration of *Perna perna* fed with *Chaetocerus gracilis* decresased significantly from 0.7 ml O_2 $g^{-1}h^{-1}$ to 0.4 ml O_2 $g^{-1}h^{-1}$ after ten days under laboratory conditions (Resgalla *et al.*, 2006). In both cases above, there was a decrease in respiration followed by a loss of dry weight which introduced greater variability in the oxygen consumption data after thirty days. This suggests that the mussels were unable to acquire enough energy to cover the basic metabolic requirements. Similar results were also obtained in studies which showed that the oxygen consumption rates of both *Mytius californianus* and *Nucella ostrina* were lower for unfed laboratory held animals compared to field specimens as well as specimens which had been kept in the laboratory and fed (Dalhoff *et al.*, 2002).

In the current study, we did not measure the rate of respiration in the field but we did so immediately after collection from Taylors Mistake. Marine bivalves such as *Mytilus edulis* have shown a decline in respiration rates after ten days during summer and after twenty five to thirty days during winter (Bayne, 1973a). During this time, the mussels increased their reliance on complex carbohydrate in tissue in the mantle and digestive glands and were thought to partially enter an anaerobic metabolic pathway (Bayne, 1973a). This pathway is accompanied by shell closure suggesting that pumping and clearance rates became zero at some periods during laboratory storage or at times when it was unfavourable for mussels to filter normally. The implications of this cascade of activity are that mussels which are held in laboratory may experience a decline in respiration rate which would induce shell closure and a decline in clearance rate.

Effects of two months storage

For mussels kept for two months, the difference in respiration rates between fed and unfed mussels was found to be significant. In addition to the effects of feeding, the trend of decreasing respiration rates as the experiment progressed was also noted as respiration rates became zero or very near zero on two occasions. These inconsistent rates of respiration rates are consistent with periodic valve closure. However, these rates may have also been an artefact because, although the water used in respirometry was changes in between evaluations, mussels faeces may have settled and accumulated in the respirometers over the successive eight sampling points. The biochemical oxygen demand (BOD) created by this refractory material in individual respirometers may have contributed to the large background respiration which was not detected by the controls. This would concur with other previous studies which suggest that suggests that increasing error when respirometry on *P. canaliculus* is done for extended periods (Waite, 1989).

Source of mussels

Results from our experiment showed that there were no significant differences between the 0.10 ± 0.007 ml O_2 g^{-1} h^{-1} recorded for intertidal and 0.08 ± 0.007 ml O_2 $g^{-1}h^{-1}$ recorded for farm *P. canaliculus* over twenty eight hours. Our results also showed that farm mussels in the 7.5-8 cm length class had significantly higher condition indices and dry weights. Because the dry weights were not similar, we could not conclude whether the consistent numerically lower respiration rate recorded for the farm mussels could be explained by a factor other than the inverse relationship between dry weight and respiration. However, the lower respiration on a dry weight basis displayed by farm mussels may be one reason why, after the juvenile phase of high growth, farm mussels were able to allocate proportionally more of their intake of energy into increasing their dry weight. This experiment also showed that the effects of time on respiration rate were not significant over the twenty-eight hours period for either the farm or wild mussels.

Only a few studies have compared the respiration rates of cultivated and naturally occurring mussels simultaneously (Labarta *et al.*, 1997). This previous study showed that respiration of intertidal mussels decreased from 0.46 ml O_2 $g^{-1}h^{-1}$ to 0.35 ml O_2 $g^{-1}h^{-1}$ over fifteen days whilst the respiration rates of raft cultivated mussels declined from 0.40 ml O_2

$g^{-1}h^{-1}$ to 0.36 ml O_2 $g^{-1}h^{-1}$. The significant effect of time which was detected probably reflected the larger decline in the intertidal mussels (Labarta *et al.*, 1997).

Effects of mass

In our experiment we obtained a value of 0.536 (r^2 = 0.8736) for the allometric exponent for respiration rate of *P. canaliculus*. This was similar to 0.535 obtained for *P. canaliculus* in May 1997 (James *et al.*, 2001) but higher than the 0.375 obtained in September 1997 (James *et al.*, 2001). The values derived for both "a" and the exponential exponent were below values derived for *M. edulis* suggesting that respiration in *P. canaliculus* is less mass dependent than *M. edulis*. Part of the reason why estimates of a and b in the first experiment were low may be because the size range of mussels was too restrictive. The effect of mass on the metabolic rate was investigated because it can contribute to variability in physiological rates. This mass-rate relationship was cited as early as 1948 in research into the chemical control of snails which were carriers of *Schistasoma mansoni* (Brand *et al.*, 1948). This early research concluded that oxygen consumption of the freshwater *Australorbis glabratus* decreased with increasing size if expressed per unit weight but that it remained constant if expressed relative to weight$^{2/3}$.

Time of day effects

There was a trend of decreasing respiration over the course of the day (Figure 3.4 & 3.9). In the first of these experiments, the statistical test failed to show significant effects in time but this may have been the result of a very small sample size (n = 2). When the sample size was increased to (n = 4) in experiment four, an effect of time was detected. Likewise, in experiment five, when respiration in fed and unfed mussels was monitored for forty eight hours, the initial oxygen consumption was high for the fed mussels but this declined by the second reading. This decline was to values that approached zero on day two of the experiment.

This difference in respiration rate at different times of day in both cases suggest either (1) elevated rates of respiration after overnight settling of mussels in the respirometry system or (2) a gradual decline in respiration through the day. The latter explanation is more

likely as the large variance in respiration in the second measurement of the day suggests that the mussels may be undergoing some form of transition from initially high values to reduced levels of oxygen consumption later in the day. These effects are not likely to be based on circadian rhythms but are more likely to be the result of handling. This conclusion is drawn because it has previously been shown that by keeping marine species under laboratory conditions, the circadian and tidal rhythms may be removed after some time (Northcott *et al.*, 1990; Kim *et al.*, 1999).

Specific dynamic action

The specific dynamic action which is characterized by a significant rise in post-feeding oxygen consumption is an approximation of the metabolic cost associated with feeding (Guillaume *et al.*, 2001). This cost can represent as much as forty seven percent of the assimilated calorific value of food (Bayne and Scullard, 1977). Previous research into the characteristics of the specific dynamic action of invertebrates have shown that it comprises aspects of the mechanical cost of feeding such as filtration (Bayne and Scullard, 1977) and gut passage, as well as the physiological costs of absorption (Kiørboe *et al.*, 1985) and catabolism of nutrients, and protein synthesis. Some researchers have also identified behavioural responses to feed stimuli and disturbance by the experimenter as important factors resulting in increased respiration rates in bivalves under study (Carefoot, 1990a). The SDA can be considered an important source of variation in bivalve energetic studies because in cases such as with the isopod *Ligia pallasii* and the copepod *Acartia tonsa*, the SDA was between 7-17% of the total energy acquired by the animals (Kiørboe *et al.*, 1985; Carefoot, 1990a). In the copepod, the post-feeding oxygen consumption respiration rate was four times that of starved animals whilst in the isopod, the consumption of oxygen increased 150% within one hour of feeding. Results from the present study indicate that immediately after feeding, oxygen consumption in *Perna canaliculus* increased from 0.15 ml O_2 $g^{-1}h^{-1}$ to 1.03 ml O_2 $g^{-1}h^{-1}$. The rise was much greater than the rise in oxygen consumption from 0.43 ml O_2 $g^{-1}h^{-1}$ before feeding to 0.51 ml O_2 $g^{-1}h^{-1}$ after feeding *P. canaliculus* with toxic *Alexandrium tamarense* (Marsden and Shumway, 1992a). However, an increase in oxygen consumption of this magnitude was seen within 20-60 minutes of feeding of *Nassarius reticulates.* This gastropod reacted to food by first extending its proboscis in the search for food (Crisp *et al.*, 1978). In the current study, this

type of behaviour was commonly seen in individuals of *P. canaliculus* which extended their feet, moved and oriented themselves in containers during feeding experiments lasting for more then a few hours. The behaviour seen in these preliminary experiments was one reason for later devising a standardized protocol which would minimize handling of the mussels in future experiments. This was because mussels tended to stop feeding for a period after being disturbed. During this period, the mussels would orient or attach themselves then start feeding again.

The duration of the SDA in the current experiment was very short in comparison to the SDA noted for *Mytilus edulis* (Bayne and Scullard, 1977). In that study, there was 100% increase in oxygen consumption immediately after feeding. This elevated respiration rate returned to pre-feeding levels after 10-32 hours. In our experiment, the reversion occurred only after two hours. The authors of that previous study state that the *M. edulis* specimens used in those experiments were kept in laboratory for between nineteen and thirty days without feed whilst the *Perna canaliculus* in the current experiment were fed a ration of fresh *T. chuii*. This differences in SDA response may be because *P. canaliculus* had become accustomed to unialgal cultures of *T. chuii* and were able to quickly react to an opportunity to feed. This contrasts with *M. edulis* which were never exposed to the feed algae after collection from the field (Bayne and Scullard, 1977).

The SDA in mussels is said to occur only after twenty four hours and is said to involve de-amination of the feed and protein synthesis (Bayne and Scullard, 1977). This high level of respiration lasted between only one and two hours suggesting that this elevated level of oxygen consumption may represent the combined effects of active metabolism and the true SDA (Thompson and Bayne, 1972). High levels of oxygen consumption representing an SDA of four times the standard metabolism has been recorded for zebra mussels provided with elevated feed and decreased inorganic sediments (Madon *et al.*, 1998). This could be an important source of variation in the study of physiological rates of *P. canaliculus*.

Conclusions

These experiments point to some critical methodologies which can be used to minimize variation and record a stable metabolic rate. Firstly, results from the first two experiments suggested that brief periods of storage of mussels resulted in a decline in the metabolic

rates of intertidal mussels. These results concur with past research which suggests that although mussels can retain a significant amount of "ecological memory" of previous ecological conditions (Mallet *et al.*, 1987), experiments aiming to discern the effects of pollutants need to be organized so that experiments use only recently collected mussels (Widdows *et al.*, 1995). In addition, even when recently harvested mussels are used, respiration in mussels is sensitive to either handling or the artificial environment in respirometers. Feeding of mussels with fresh cultures was found to keep respiration at rates significantly higher than the unfed mussels. This was however accompanied by erratic respiration. Unfed mussels had a low but stable respiration rate. When recently harvested naturally occurring or cultivated *P. canaliculus* were used as experimental subjects, the differences between the mussels of different origin were not significant and respiration rates were stable. This suggests that the metabolic rates which accompany freshly collected farm or intertidal mussels should be similar at the start of experiments.

Chapter 4 Effects of copper on clearance and respiration rates of intertidal and farm *Perna canaliculus*

4.1 Introduction

The use of mytilid bivalves as bioindicators of low concentrations of highly toxic contaminants in natural waters has developed steadily from its inception in the 1960's. The original research in this field focused on chemical analyses of the tissues to identify deviations from baselines which were used for environmental protection (Arnold *et al.*, 2006) and food safety purposes. A number of the environmental protection efforts quickly evolved into global programmes which led to the identification of numerous global sites where persisting organic pollutants (POPs) or trace metal contamination had occurred and were in need of remediation. Recent experiences with endocrine disruptors such as tributyltin (TbT) (Horiguchi *et al.*, 2006) and trace metals such as copper (Jeng *et al.*, 2000) also suggest that monitoring of industrial contaminants will continue to require scientific progress in the field of aquatic toxicology. This is because, in both the TbT and copper pollution cases, the environmental risks and effects of these compounds on marine species were discovered well after considerable exposure to the compounds had occurred (Alzieu, 2000). These cases point to one of the inherent weaknesses of retrospective techniques which use bivalves as sentinels – the issue of the extended period of exposure that is required to detect changes in the concentration of toxins in mussel tissues. Even though there has been considerable research conducted to refine methods to understand the environmental physiology of bivalves (Underwood and Peterson, 1988), there is a need to develop new techniques for quantifying physiological responses in bivalves used as

biomonitors (Widdows and Johnson, 1988). Recent advances in marine ecophysiology include improved methods for measuring physiological indices such as clearance rate (Filgueira *et al.*, 2006; Elliott *et al.*, 2008) which is used in the calculation of Scope for Growth (SfG). In addition, new research fields which investigate the associations between metal contaminants and the physiology and behaviour of bivalves are being developed (McDowell *et al.*, 1999; El-Shenawy, 2004).

There is only limited information on the effects of copper on *P. canaliculus* and other species of mussels in New Zealand. The present experiments were used to develop techniques to investigate changes in the energetics of *Perna canaliculus* when exposed to copper at concentrations of parts per billion (μg Cu L^{-1}). Specifically, these experiments quantified the effects of copper on the clearance, oxygen consumption and excretion rates of *P. canaliculus* collected from subtidal farm ropes and intertidal rocks. The effects of copper on the physiological indices of *P. viridis* and *M. edulis* have been studied before but only limited research has been conducted on the sublethal effects of copper on the New Zealand species. The research was developed because *Perna canaliculus* is one the most proficient bivalve filter feeders of New Zealand's coastal waters and its physiology could be a good indicator of the properties of these waters. The four experiments which compared the effects of copper on clearance, respiration and excretion on intertidal and farm mussels were conducted between September 2006 and January 2007. The first experiment tested the effects of concentrations of 0 – 100 μg Cu L^{-1} on intertidal mussels. The second and third experiments tested effects of 0 – 1,000 μg Cu L^{-1} exposures on farm an intertidal specimens. The final experiment tested effects of four concentrations ranging from 0 – 500 μg Cu L^{-1} on intertidal specimens. The aim was to test whether differences in the clearance, respiration, excretion or scope for growth (SfG) could be determined after exposure to various concentrations of copper for various lengths of time.

4.2 Methods

List of experiments

Four experiments which investigated the physiological effects of copper on intertidal and farm mussels were conducted between September 2006 and January 2007:

Experiment 1: Intertidal mussels exposed to 0 μg Cu L^{-1}, 1 μg Cu L^{-1}, 10 μg Cu L^{-1} and 100 μg Cu L^{-1}.

Experiment 2: Farm mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1}.

Experiment 3: Intertidal mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1}.

Experiment 4: Intertidal mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 250 μg Cu L^{-1} and 500 μg Cu L^{-1}.

Experiment 1 (September 25, 2006): Intertidal mussels exposed to 0 μg Cu L^{-1}, 1 μg Cu L^{-1}, 10 μg Cu L^{-1} and 100 μg Cu L^{-1}.

Experimental design

Mussels were exposed to treatments of 0 μg Cu L^{-1}, 1 μg Cu L^{-1}, 10 μg Cu L^{-1} and 100 μg Cu L^{-1} at a salinity of 34 ppt. A total of twenty-five mussels were stocked in each exposure tank at the start of the experiment. A sample of n = 4 mussels was collected after 1, 3, 7, 14 and 21 days of exposure and clearance and respiration rates were measured on these mussels as in sections 2.6 and 2.7.

Collection and maintenance

The one hundred *P. canaliculus* mussels (shell length 6-7 cm) used in this experiment were collected on September 14, 2007 at a location south of the bay at Taylors Mistake, Christchurch. The mussels were collected by cutting the byssus threads and removing the mussels from intertidal rocks during low tide. These mussels were then transported in plastic buckets to the SBS aquarium room where they were brushed and scraped cleaned of

barnacles, epiphytes and algae. The mussels were then randomly placed in three 40 L aerated aquaria in recirculating seawater. These mussels were not attached to perspex plates or any other substrate and they formed byssal attachments to the sides and bottom of the exposure tanks. After ten days, all mussels were collected from the three aquarium tanks and transferred to the temperature control room on level 6 at the SBS where the eight tanks in the gravity flow exposure system were located (Figure 2.7).

Experimental setup and evaluation

Twenty-five mussels were placed individually in each of the four exposure tanks. One tank was used as a control without added copper and the other three tanks were treatments tanks spiked with aqueous copper chloride. Tanks two, three and four contained seawater spiked with copper chloride at a concentration of 1, 10 and 100 μg Cu L^{-1} respectively.

The stock solution for the 1 μg Cu L^{-1} treatment was made by dissolving 0.0938 g $CuCl_2.2H_2O$ into 1,000 ml dH_2O. One ml of this stock solution was applied to tank two to achieve 1 μg Cu L^{-1}. The second stock was made by placing 9.38 g $CuCl_2.2H_2O$ into 1,000 ml dH_2O. In order to achieve 10 μg Cu L^{-1} and 100 μg Cu L^{-1}, 0.1ml and 1 ml of the second stock solution were added to tanks three and 1 ml was added to tank four.

Mussels in all four tanks were fed fresh *Isochrysis galbana* daily. After 1, 3, 7, 14 and 21 days of exposure, a sample of 4 mussels per tank was removed and used to determine clearance rate and oxygen consumption as in section 2.6 and 2.7.

Experiments 2 and 3 (8 November 2006, 26 November, 2006): Farm and intertidal mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1}.

Experimental design

Twenty five farm and intertidal mussels were exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} in experiments two and three respectively. After three, seven and fourteen days of exposure, n = 4 mussels were removed and evaluated for clearance and respiration as in section 2.6 and 2.7. Statistical analysis was conducted as in

section 2.10 in order to determine if differences in clearance or respiration could be detected among the different treatments.

Collection and maintenance

The farm mussels used in experiment two were from an aquaculture facility in Pigeon Bay. These were delivered to the SBS on the same day they were collected by the farmer. Mussels for experiment three were sourced from Taylors Mistake. In both cases, mussels were cleaned of barnacles and individually labeled at the umbo region using a 2 mm^2 numbered tag made from metallic ribbon tape. Mussels were not fed before the experiments.

Experimental setup and evaluation

The two experiments evaluating the effects of 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} on farm and intertidal mussels were started on the 8 th. and 26 th. November in 2006 respectively. All mussels were stocked on the same day for these two consecutive experiments. For each experiment, the exposure water was changed daily but mussels were not fed for either experiment. Both experiments utilized the flow through exposure system described in section 2.5

In each experiment, twenty-five mussels were stocked per tank. After this, the copper solution was applied from stock solutions to the header and exposure tanks. The 100 μg Cu L^{-1} stock was made using 0.992 mg $CuCl_2.2H_2O$ dissolved in 100 ml of distilled water. The stock for the 500 μg Cu L^{-1} treatment was made from 4.96 mg $CuCl_2.2H_2O$ dissolved in 100 ml distilled water. This stock was also used for the 1,000 μg Cu L^{-1} treatment.

The header tank in each system was spiked with the appropriate amount of copper stock and fresh seawater was added on a daily basis before each exposure system was allowed to exchange by gravity flow over the subsequent 20 hours.

Individual tanks were checked for dead mussels at the time of addition of fresh seawater in the mornings and the dead mussels were removed and discarded. Mussels were not fed for

these experiments. On days three, seven and fourteen, n = 4 mussels were removed from each tank by cutting the byssus under water. The mussels were then placed in containers described in 2.6 and 2.7 and the clearance and respiration were measured over three hours.

Experiment 4 (25 January, 2007): Intertidal mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 250 μg Cu L^{-1} and 500 μg Cu L^{-1}.

Experimental design

Experiment four was planned and implemented using a standard operating procedure developed after analysing data from experiments one, two and three. The standard operating procedure ensured that all subsequent experiments were started within 5 days of collection of the mussels. The operating procedure provided for a sample size of eight mussels to be exposed to four treatments. Evaluation of treatment effects were conducted after three, seven and fourteen days. Mussels could be sampled daily for survival without excessive handling. The experiences of the previous experiments had shown that clearance, respiration and excretion could be determined on a maximum sixteen mussels on a daily basis. As a result, the eight replicate mussels for each time interval were stocked on a staggered basis over two days. Sampling after three, seven and fourteen days of exposure was then conducted over two days, in accordance with the order that mussels were stocked. Also, before mussels were collected for the experiment, algal cultures were harvested, concentrated and frozen to ensure availability of feed for the mussels during the exposures.

Collection and maintenance

Two hundred mussels (shell length 5-7 cm) were cut from the intertidal rocks during low tide on January 24, 2006 at Taylors Mistake. Taylors Mistake is considered an optimal sampling site because it is south of Christchurch City, away from industrial influences. These mussels were then transported in plastic buckets to the University of Canterbury School of Biological Sciences (SBS) aquarium room.

The mussels were place in the SBS recirculating aquarium facility and held at 13°C. One day after collection, the mussels were brushed and scraped cleaned of barnacles, epiphytes and algae. A numbered plastic tag (≈ 3mm x 2mm) was attached to each mussel at the umbo region using cyanoacrylate glue. Each mussel was then attached to a 5 cm x 5 cm perspex plate with cyanoacrylate glue. These were then temporarily suspended in flowing seawater in three 20 L buckets. The mussels were then kept suspended in the buckets with their siphons facing upward and water was flushed through the buckets for 30 minutes. After 30 minutes, the flow of water was reduced and the buckets were kept gently flushing in order to remove any trace of the cyanoacrylate glue. Gentle flowing of water into the buckets was continued for two days. During this time, the exposure system was filled and aeration and protein skimmers were placed into the systems (Figures 2.7 & 2.8).

Experimental setup and evaluation

The mussels used in this experiment were stocked on a staggered basis into the experimental system over two days starting on January 25, 2007. Stocking was achieved by suspending the attached mussels into the tanks using a rope tied perpendicularly to the shorter dimension of each tank (Figure 2.8). Mussels for physiological measurements were not allowed to touch the bottoms of the exposure tanks (Figure 2.9). The medium used in this experiment was 34 ppt salinity unfiltered seawater maintained at 13°C at the aquarium facility holding tank. Each morning at 6:00-7:00 AM, 148 L of seawater was transferred from the aquarium supply tank to the exposure tanks so that 37 L of seawater was added to each header tank per day. An equal amount of water was then discarded from the waste water collection tanks (Figure 2.7). After the 37 L of fresh seawater was added to header tanks on each day, each header tank was treated with a copper stock solution to give treatment concentrations of 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 250 μg Cu L^{-1} and 500 μg Cu L^{-1}. The stock used for the 100 μg Cu L^{-1} treatment was made of 1.984 mg $CuCl_2.2H_2O$ in 200 ml water. The other stock used was made of 4.96 mg $CuCl_2.2H_2O$ dissolved in 100 ml distilled water. One ml of this stock was used for the 500 μg Cu L^{-1} treatment and 0.5 ml was used for the 250 μg Cu L^{-1} treatment.

Each day, mussels were fed with thawed out *Tetraselmis chuii* at an estimated rate of 2% of the estimated dry weight. After the third day of exposure, the first group of four

mussels (n = 4) was removed from each tank and the clearance, respiration and excretion rates were determined starting at 9 am and finishing at 7 pm. On the next day, the second group of (n = 4) mussels was sampled for determination of the clearance, respiration and excretion rates. Data were compiled and analysed as in section 2.10.

4.3 Results

Experiment 1: Intertidal mussels exposed to 0 µg Cu L^{-1}, 1 µg Cu L^{-1}, 10 µg Cu L^{-1} and 100 µg Cu L^{-1}

The mean clearance rates determined in this experiment varied considerably over the 21 days of exposure with low values for day one followed by high values for day three and seven and a return to low values on day fourteen (Figure 4.1).

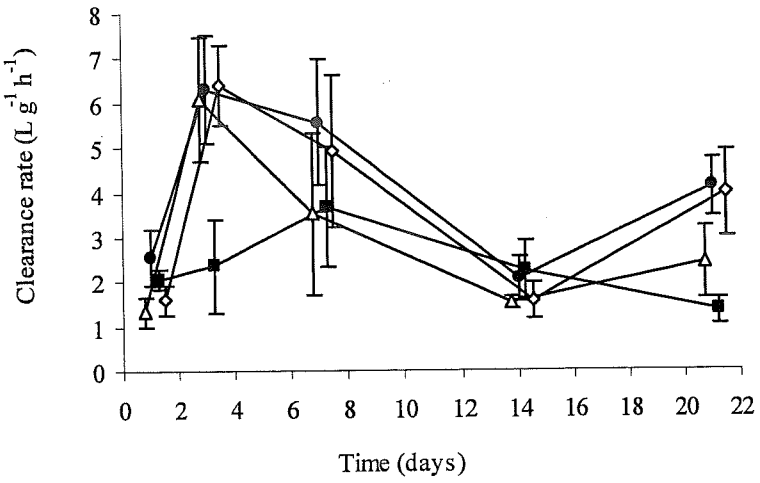


Figure 4.1: Clearance rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 1 µg Cu L^{-1}(■) , 10 µg Cu L^{-1} (△) and 100 µg Cu L^{-1}(●) after one, three, seven, fourteen and twenty-one days of exposure.

Table 4.1: Repeated measures ANOVA of clearance rates of intertidal mussels exposed to 0 μg Cu L^{-1}, 1 μg Cu L^{-1}, 10 μg Cu L^{-1} and 100 μg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	0.07	3	0.02	6.87	0.006
Duration of exposure	0.25	4	0.06	8.78	<0.001
Copper concentration*Duration of exposure	0.082	12	0.007	0.962	NS

Analysis of the clearance data for the 0 and 100 μg Cu L^{-1} treatments using repeated measures ANOVA showed that there were differences based on copper exposure as well as differences among the five sampling days (Table 4.1). The significant differences in clearance which the repeated measures ANOVA detected were investigated using *post hoc* Tukeys HSD test which confirmed that clearance recorded for the 1 μg Cu L^{-1} treatments (2.32 L g^{-1} h^{-1}) were significantly lower than the controls (3.68 L g^{-1} h^{-1}). The *post hoc* test also revealed that the significant effect of duration of exposure was because the clearance rates on days three and seven were significantly higher than the rest of the days.

ANOVA of clearance rate values for individual days shows that there were no significant differences based on copper concentration on day one (ANOVA $F_{(3,15)} = 1.24$, $p = 0.33$), three (ANOVA $F_{(3,15)}$, $F = 3.24$,$p = 0.06$), seven (ANOVA $F_{(3,15)} = 0.41$,$p = 0.75$) and fourteen (ANOVA $F_{(3,15)} = 0.63$, $p = 0.61$). However, significant differences were detected on twenty one (ANOVA $F_{(3,15)} = 3.65$, $p=0.04$) when clearance for the 1 μg Cu L^{-1} treatment was found to be lower than for the controls and the 100 μg Cu L^{-1} treatments.

Respiration rate

There was considerable variability in respiration rate among the different treatments and there were no distinctive trends among the treatments (Figure 4.2).

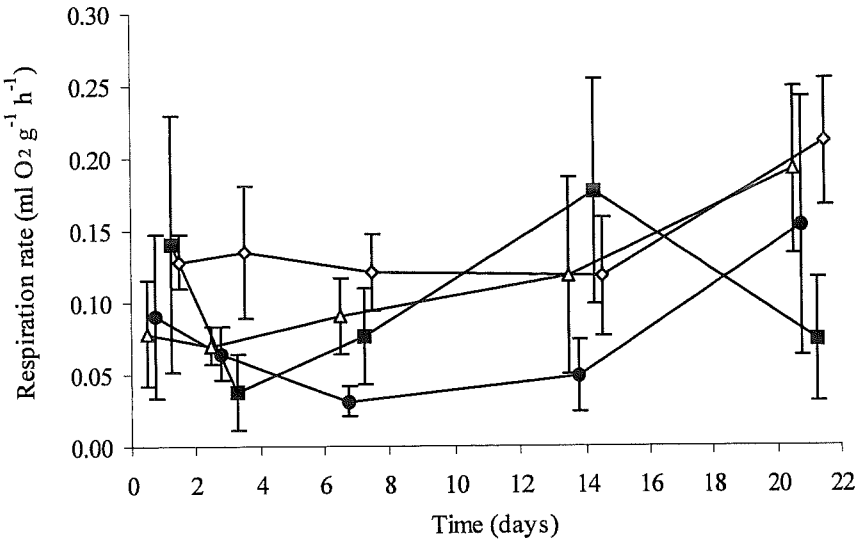


Figure 4.2: Respiration rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 1 µg Cu L^{-1}(■) , 10 µg Cu L^{-1} (△) and 100 µg Cu L^{-1}(●) after one, three, seven, fourteen and twenty-one days of exposure.

The highest average respiration rate was for the control which respired at a rate of 0.14 ± 0.03 ml O_2 g^{-1} h^{-1}. The lowest respiration was for the 100 µg Cu L^{-1} treatment which was 0.08 ± 0.04 ml O_2 g^{-1} h^{-1}. The rates for the other two treatments were intermediate between these values. As a result, analysis with repeated measures ANOVA showed that no significant differences could be detected based on the four concentrations of copper exposures or differences over the five time intervals (Table 4.2).

Table 4.2: Repeated measures ANOVA of respiration rates of intertidal mussels exposed to 0 µg Cu L^{-1}, 1 µg Cu L^{-1}, 10 µg Cu L^{-1} and 100 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	<0.001	3	0.00	0.79	NS
Duration of exposure	<0.001	4	0.00	2.39	NS
Copper concentration*Duration of exposure	<0.001	12	0.00	0.95	NS

Comparing the mean respiration for each of the days using ANOVA showed that there were no significant differences on day one (ANOVA $F_{(3,15)} = 0.28$, $p = 0.84$), three (ANOVA $F_{(3,15)} = 2.04$, $p = 0.16$), seven (ANOVA $F_{(3,15)} = 2.20$, $p = 0.14$), fourteen (ANOVA $F_{(3,15)} = 0.83$, $p = 0.50$) or day twenty one (ANOVA $F_{(3,15)} = 0.98$, $p = 0.43$).

Condition index

The average condition of the four treatment groups declined gradually from 98.01 on the first day of the experiment to 72.14 on the last day of the experiment (Figure 4.3).

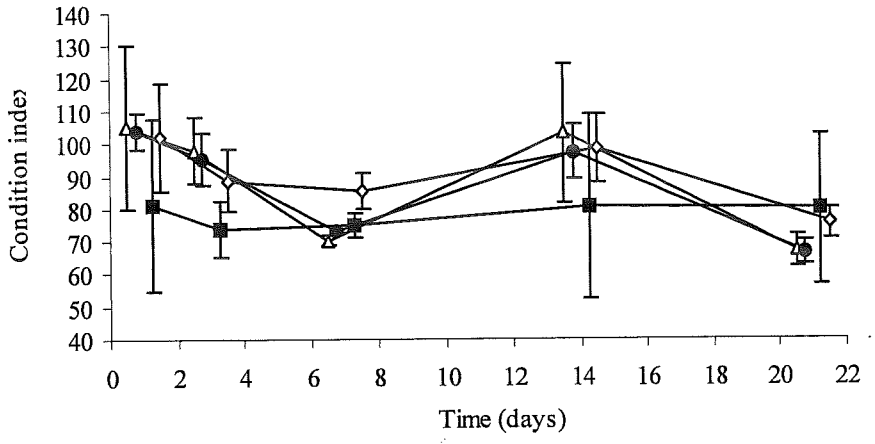


Figure 4.3Condition indices +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 1 µg Cu L^{-1}(□) , 10 µg Cu L^{-1} (△) and 100 µg Cu L^{-1}(●) after one, three, seven, fourteen and twenty-one days of exposure.

There was considerable variability among the treatments at each time interval. On the first day of sampling, the 0 µg Cu L^{-1}, 1 µg Cu L^{-1}, 10 µg Cu L^{-1} and 100 µg Cu L^{-1} treatments recorded conditions of 102, 81, 105, 103 respectively. At the end of the experiment, the 21 day average for the 0 µg Cu L^{-1}, 1 µg Cu L^{-1}, 10 µg Cu L^{-1} and 100 µg Cu L^{-1} treatments were 90, 77, 88 and 87. Analysis of the data using repeated measures ANOVA showed that the trend of slowly declining condition in most of the animals did not constitute a statistically significant loss of condition based on the concentration of copper exposure or because of the duration of the exposure (Table 4.3).

Table 4.3: Repeated measures ANOVA of condition index of intertidal mussels exposed to 0 µg Cu L^{-1}, 1 µg Cu L^{-1}, 10 µg Cu L^{-1} and 100 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	0.34	3	0.11	1.57	NS
Duration of exposure	0.53	4	0.13	1.74	NS
Copper concentration*Duration of exposure	0.37	12	0.03	0.41	NS

Experiment 2: Farm mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1,000 µg Cu L^{-1}.

Mortality and clearance rate

All mussels stocked in the experiment had survived on day three of the exposures but by day seven, all the mussels in the 500 µg Cu L^{-1}, and 1,000 µg Cu L^{-1} treatments had died. None of the mussels in the controls or the 100 µg Cu L^{-1} treatment had died and clearance rates for these two groups were similar over the duration of the experiment (Figure 4.4).

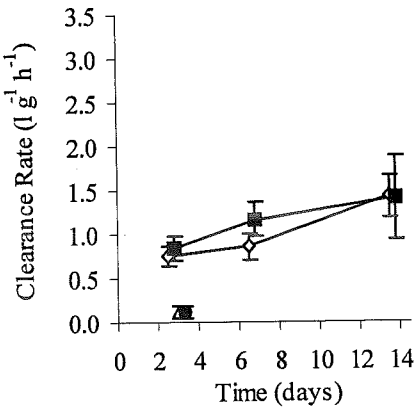


Figure 4.4: Clearance rate +/- SE of farm mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■) , 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

However, these clearance rates were considerably higher than rates recorded for mussels exposed to 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} on day three. Because all mussels in the 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} had died by day seven, only comparisons between the 0 μg Cu L^{-1} and the 100 μg Cu L^{-1} were possible for the full course of the experiment.

Comparison of these data using repeated measures ANOVA revealed that there were no statistically significant differences between the controls and the mussels exposed to 100 μg Cu L^{-1}. Also, there were no differences in clearance among the different time intervals when the evaluations were conducted after three, seven and fourteen days of exposure (Table 4.4). No significant interactions were detected.

Table 4.4: Repeated measures ANOVA of clearance rate of farm mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1000 μg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	5.92	2	2.96	10.20	NS
Duration of exposure	2.62	2	1.31	4.50	NS
Copper concentration*Duration of exposure	1.35	2	0.67	2.3	NS

This result occurred because the 0 μg Cu L^{-1} and 100 μg Cu L^{-1} treatments showed similar patterns over the course of the experiment. During the interval between days three and fourteen, clearance of the control mussels increased from an average of 0.75 ± 0.12L g^{-1} h^{-1} to 1.42 ± 0.24 L g^{-1} h^{-1}. The 100 μg Cu L^{-1} exposed mussels displayed a similar pattern of increasing clearance from 0.83 ± 0.14 L g^{-1} h^{-1} to 1.41 ± 0.48 L g^{-1} h^{-1} between days three and fourteen.

Respiration rate

Respiration rates for mussels in the 0 μg Cu L^{-1} and 100 μg Cu L^{-1} treatments were stable over the course of this experiment (Figure 4.5). However, respiration for the mussels exposed to 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} were much lower than rates for the control and 100 μg Cu L^{-1} treatments on day three (Fig. 4.5).

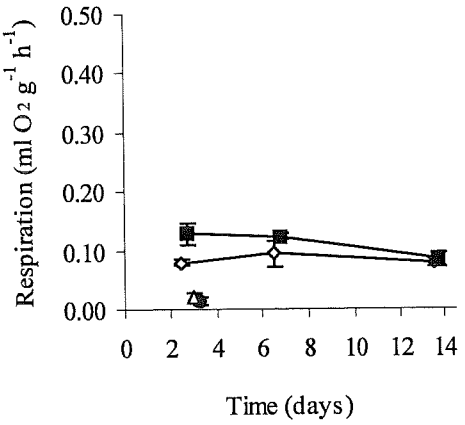


Figure 4.5: Respiration rate +/- SE of farm mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■) , 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Analysis of the data for the controls and the 100 µg Cu L^{-1} treatments for the duration of the experiment using repeated measures ANOVA showed that no effects of exposure to copper were detected between the two treatments. Also, no effect of time was seen in the respiration rates measured at the three time intervals (Table 4.5).

Table 4.5: Repeated measures ANOVA of respiration rate of farm mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1000 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	0.01	2	0.00	0.63	NS
Duration of exposure	0.02	2	0.01	1.51	NS
Copper concentration*Duration of exposure	0.01	2	0.00	0.57	NS

The lack of significant differences due to exposure to copper was in spite of the fact that the differences among the day three respiration rates were significant (ANOVA $F_{(3,12)} = 22.35$, $p < 0.001$) significant. *Post hoc* evaluation of this result confirmed that the

differences between respiration of the controls and the 100 µg Cu L^{-1} treatments were significant on day three.

Condition Index

The data on condition of the mussels on day three showed considerable variability with the controls and the 100 µg Cu L^{-1} treatments showing lower values than the mussels exposed to either 500 Cu L^{-1} or 1,000 µg Cu L^{-1} (Figure 4.6). After this, the condition of the controls and the 100 µg Cu L^{-1} treatments remained similar at the other two intervals when measurements were taken.

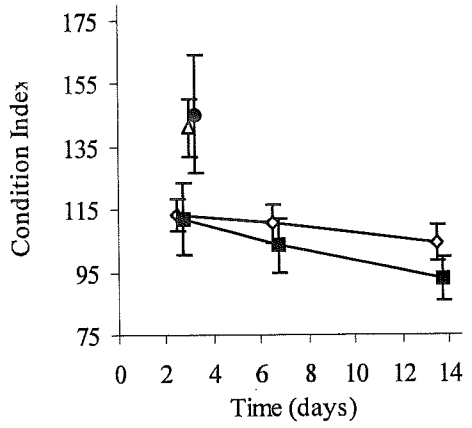


Figure 4.6:Condition index +/- SE of farm mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Analysis of the trends shown by the controls and the 100 µg Cu L^{-1} treated mussels from day three to day fourteen using the repeated measures ANOVA showed that no differences in condition in these two treatments could be attributed to concentration of copper or the duration of the exposure (Table 4.6).

Table 4.6: Repeated measures ANOVA of condition index of farm mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1000 µg Cu L^{-1}

Source of Variation	SS	df	MS	F	p
Copper concentration	367.20	2	183.60	0.84	NS
Duration of exposure	134.50	2	67.20	0.31	NS
Copper concentration*Duration of exposure	48.90	2	24.40	0.11	NS

Experiment 3: Intertidal Mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1,000 µg Cu L^{-1}.

Mortality and clearance rate

The first notable outcome of this experiment was that all mussels in the 1,000 µg Cu L^{-1} treatment died by day seven and all mussels in the 500 µg Cu L^{-1} treatment died by day fourteen. In addition to the mortalities, the clearance rates were very low for the 500 µg Cu L^{-1} and 500 µg Cu L^{-1} treatments on day three. Clearance rates measured for the 0 µg Cu L^{-1} and 100 µg Cu L^{-1} treatments were similar and stable over the fourteen day period (Figure 4.7).

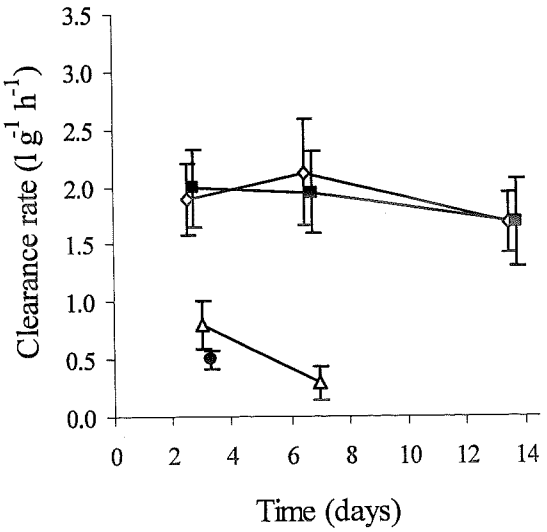


Figure 4.7: Clearance rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Analysis of the data with repeated measures ANOVA showed that there were no differences in clearance rates between the controls and the mussels in the 100 µg Cu L^{-1} exposures (Table 4.7). The effects of duration of exposure or interactions between exposure to copper and the duration of exposures were not significant (Table 4.7).

Table 4.7: Repeated measures ANOVA of clearance rate of intertidal mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1000 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	2.62	2	1.31	4.50	NS
Duration of exposure	1.35	2	0.67	2.3	NS
Copper concentration*Duration of exposure	0.27	2	0.14	0.5	NS

Respiration rate

Data presented in Figure 4.8 shows that there was considerable variability among the treatments in terms of the oxygen consumption recorded on day three. The variability among the surviving treatments decreased on day seven. By day fourteen, respiration in the controls and the 100 µg Cu L^{-1} treatments were very similar.

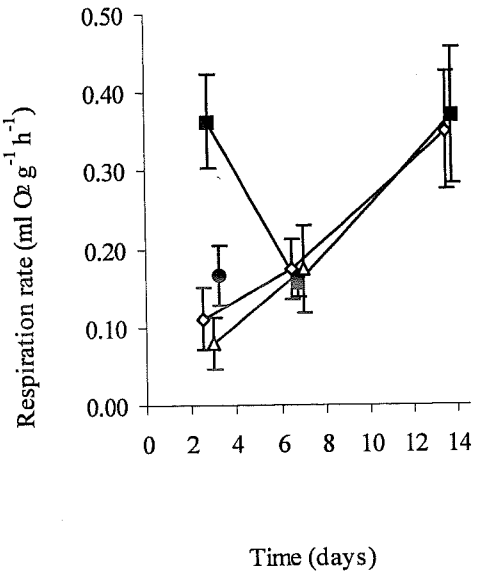


Figure 4.8: Respiration rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

The respiration rate of the 100 μg Cu L^{-1} treatment was high on day three. Although the rates for the 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} treatments were low at this time, they were within range of the controls and did not indicate severe metabolic impairment of the mussels exposed to these treatments on day three. The rate for the controls increased from 0.11 ± 0.04 ml O_2 g^{-1} h^{-1} on day three to 0.35 ± 0.07ml O_2 g^{-1} h^{-1} on day fourteen.

These results for the 0 μg Cu L^{-1} and 100 μg Cu L^{-1} treatments were analysed with a repeated measures ANOVA (Table 4.8) which showed that there was no difference in oxygen consumption between these two groups which could be attributed to exposure to copper. However, the repeated measures ANOVA showed an effect of duration of exposure (Table 4.8).

Table 4.8: Repeated measures ANOVA of respiration rate of intertidal mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 500 μg Cu L^{-1} and 1000 μg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	0.02	2	0.01	1.51	NS
Duration of exposure	0.01	2	0.00	0.57	0.020
Copper concentration*Duration of exposure	0.02	2	0.01	1.14	NS

The significant effect of duration of exposure was evaluated by *post hoc* Tukeys test which showed that oxygen consumption on day fourteen was significantly higher than on day seven.

Condition index

For the duration of this experiment, the condition determined for the controls was stable but the condition determined for the 100 μg Cu L^{-1} and 500 μg Cu L^{-1} treatments showed some variability over time (Figure 4.9).

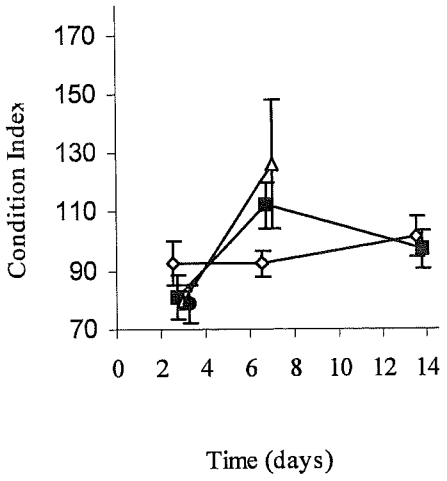


Figure 4.9: Condition index +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

When the compiled condition data for the 0 µg Cu L^{-1} and 100µg Cu L^{-1} treated mussels were evaluated using repeated measures ANOVA, no significant differences could be attributed to copper exposure or the duration of the exposures. Likewise, no interactions between copper exposure and duration of exposure were detected (Table 4.9).

Table 4.9: Repeated measures ANOVA of condition index of intertidal mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1000 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	134.50	2	67.20	0.31	NS
Duration of exposure	48.90	2	24.40	0.11	NS
Copper concentration*Duration of exposure	389.20	2	194.60	0.89	NS

Experiment 4: Intertidal Mussels exposed to 0 μg Cu L^{-1}, 100 μg Cu L^{-1}, 250 μg Cu L^{-1} and 500 μg Cu L^{-1}.

Survival/mortality

The percentage survival of the twenty-four intertidal *Perna canaliculus* held for survival analysis is illustrated in Figure 4.10. This shows that at the end of fourteen days of exposure, all animals that were exposed to either 0 μg Cu L^{-1} and 100 μg Cu L^{-1} had survived.

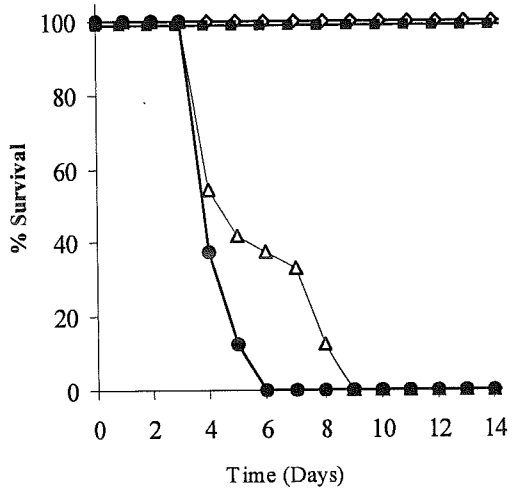


Figure 4.10: Percentage survival of *P. canaliculus* after 14 days exposure to 0 μg Cu L^{-1} (◇), 100 μg Cu L^{-1}(■), 250 μg Cu L^{-1} (△) and 500 μg Cu L^{-1}(●).

Of the mussels exposed to 500 μg Cu L^{-1}, all had survived until day three. On day four, fifteen had died. On day five, six died and on day seven, three died. At this point, all mussels subject to this treatment had died. The estimated time for 50% mortality in 500 μg Cu L^{-1} was 3.4 days. Of the 24 mussels exposed to 250 μg Cu L^{-1}, all had survived up until day three. On day four, eleven died. This was followed by a further three which died on day five. On days six and day seven, one mussel died per day. On day eight, five mussels died and on the last day, three mussels had died. After 5.15 days, 50% of the mussels in 250 μg Cu L^{-1} had died.

Clearance rate

The trends in clearance rates determined in this experiment (Figure 4.11) show that from the onset, the control mussels displayed higher clearance that the other treatments. Also, clearance rate for the controls increased over the fourteen days of the experiment.

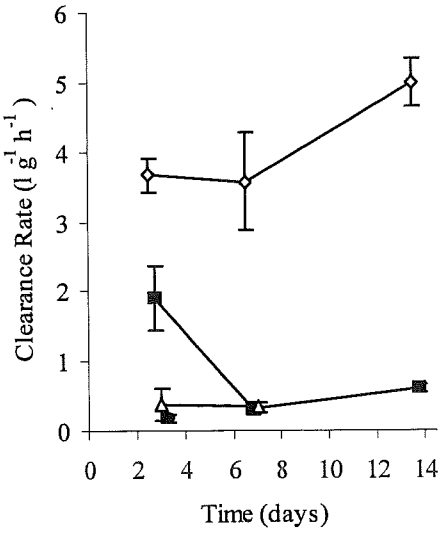


Figure 4.11:Clearance rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 250 µg Cu L^{-1} (△) and 500 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Clearance by the 100 µg Cu L^{-1} exposed mussels decreased between days three and seven and did not increase on day fourteen as the control did. The 250 µg Cu L^{-1} and 500 µg Cu L^{-1} treatments recorded very low clearance preceding 100% mortality in these two groups of mussels by days nine and six respectively.

Analysis of the clearance rates of the control and 100 µg Cu L^{-1} treatment using repeated measures ANOVA over the 14 days showed significant differences due to exposure to copper but no significant differences due to the duration of the exposure (Table 4.10). There was a significant interaction between concentration of copper and the duration of exposure (Table 4.10).

Table 4.10: Repeated measures ANOVA of clearance rate of intertidal mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 250 µg Cu L^{-1} and 500 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	117.80	1.00	117.80	129.30	<0.001
Duration of exposure	7.80	2.00	3.90	2.90	NS
Copper concentration*Duration of exposure	13.80	2.00	6.90	5.10	0.013

Post hoc evaluation of the significant effects detected by the repeated measures ANOVA confirmed that average clearance for the controls was significantly higher than clearance in the 100 µg Cu L^{-1} treatment. These results show that mussels could not increase clearance with time if kept continuously exposed to 100 µg Cu L^{-1}.

Respiration rate

Results show that respiration rates recorded for the controls was quite variable over the three time intervals. The respiration rates recorded for the 100 µg Cu L^{-1} decreased with time while the rates for mussels which were exposed to 250 µg Cu L^{-1} and 500 µg Cu L^{-1} were severely depressed (Figure 4.12).

Analysis of these respiration rates using repeated measures ANOVA (Table 4.11) indicates that over the course of the experiment, significantly lower levels of respiration were determined for mussels exposed to 100 µg Cu L^{-1}. While the effect of copper was significant, the effects of time and the time-copper concentration interaction were not significant over the fourteen days.

Post hoc examination of the significant differences detected by the repeated measures ANOVA utilizing Tukeys HSD test confirmed that the respiration rate of 0.18 + 0.05 ml O_2 g^{-1} h^{-1} for the controls was greater than 0.09 + 0.03 ml O_2 g^{-1} h^{-1} determined for the 100 µg Cu L^{-1} treatment.

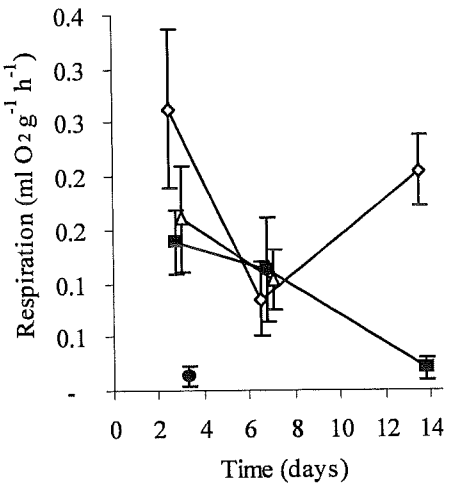


Figure 4.12: Respiration rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■) , 250 µg Cu L^{-1} (△) and 500 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Table 4.11: Repeated measures ANOVA of respiration rate of intertidal mussels exposed to 0 µg Cu L^{-1}, 100 µg Cu L^{-1}, 500 µg Cu L^{-1} and 1000 µg Cu L^{-1}.

Source of Variation	SS	df	MS	F	p
Copper concentration	0.12	1	0.12	4.99	0.042
Duration of exposure	0.05	2	0.02	2.06	NS
Copper concentration*Duration of exposure	0.10	2	0.05	4.12	0.027

Excretion

The trends depicted in (Figure 4.13) show that there were no differences in excretion between the control and the 100 µg Cu L^{-1} treated mussels but that excretion for the 250 µg Cu L^{-1} and 500 µg Cu L^{-1} exposed mussels was elevated at the start of the experiment.

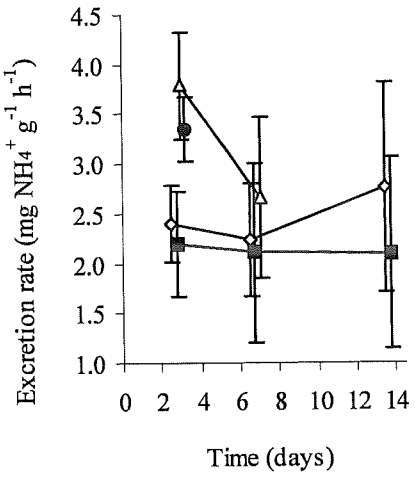


Figure 4.13: Excretion rate +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 250 µg Cu L^{-1} (△) and 500 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

Analysis of this data using repeated measures ANOVA showed that exposure to copper or the duration of the exposure caused no significant effects on the rate of excretion (Table 4.12).

Table 4.12: Repeated measures ANOVA of excretion rates of intertidal mussels exposed to 0 µg Cu L^{-1}, 100µg Cu L^{-1}, 250µg Cu L^{-1}, and 500µg Cu L^{-1} after 3, 7 and 14 days of exposure.

Source of Variation	SS	df	MS	F	p
Copper concentration	1.34	1	1.34	0.27	NS
Duration of exposure	0.54	2	0.27	0.06	NS
Copper concentration*Duration of exposure	0.65	2	0.32	0.07	NS

Scope for Growth (SfG)

Results compiled show that SfG of the controls was higher than for the other treatments and that SfG increased with time for the controls while the SfG of the mussels treated with 100 µg Cu L^{-1} declined over the fourteen days of the experiment (Figure 4.14).

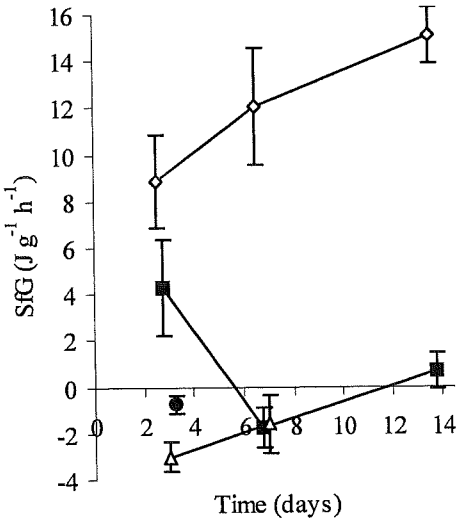


Figure 4.14: SfG +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 500 µg Cu L^{-1} (△) and 1,000 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

The SfG of the mussels in the 250 µg Cu L^{-1} treatment was initially low and showed no appreciable increase or decline between days three and seven.

Analysis of the data using repeated measures ANOVA (Table 4.13) indicated that over the course of this experiment, SfG in mussels exposed to 0 µg Cu L^{-1} and 100 µg Cu L^{-1} were significantly different. The effect of duration of exposure was not significant but the interaction between concentration of copper and duration of exposure was significant.

Table 4.13: Repeated measures ANOVA of SfG of intertidal mussels exposed to 0 µg Cu L^{-1}, 100µg Cu L^{-1}, 250µg Cu L^{-1}, and 500µg Cu L^{-1} after 3, 7 and 14 days of exposure.

Source of Variation	SS	df	MS	F	p
Copper concentration	1413.00	1	1413.00	58.87	<0.001
Duration of exposure	78.00	2	39.00	1.65	NS
Copper concentration*Duration of exposure	257.00	2	128.00	5.41	0.010

Post hoc evaluation of the significant differences detected by the repeated measures ANOVA confirmed that the average SfG of 11.97 ± 1.90 J g^{-1} h^{-1} of the controls was significantly greater than average of 1.06 ± 1.26 J g^{-1} h^{-1} for the 100 µg Cu L^{-1} treatment.

The first indication of an effect of copper on SfG was on day three when significant differences among the treatments (ANOVA $F_{(3,15)}$ =14.47, p<0.001) were noted. *Post hoc* evaluation of this effect conducted using a *post hoc* Tukeys HSD test showed that the difference between the controls and the 250 µg Cu L^{-1} and 500 µg Cu L^{-1} treatments were significant. Additionally, the difference between the control and the 100 µg Cu L^{-1} treatment was significant on day seven (ANOVA $F_{(3,15)}$ =24.69, p<0.001). *Post hoc* evaluation with Tukeys HSD test confirmed this.

Condition Index

The condition indices measured for the mussels on days three, seven and fourteen displayed in Figure 4.15 indicate that the mussels were in similar condition on day three and that this began a trend that continued to day 14 when the controls and 100 µg Cu L^{-1} mussels remained in similar condition.

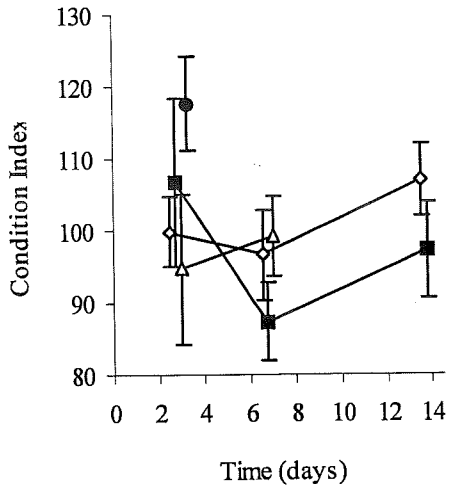


Figure 4.15: Condition index +/- SE of intertidal mussels exposed to 0 µg Cu L^{-1} (◇), 100 µg Cu L^{-1}(■), 250 µg Cu L^{-1} (△) and 500 µg Cu L^{-1}(●) after three, seven and fourteen days of exposure.

When condition of the control and 100 µg Cu L^{-1} challenged mussels were compared over the 14 days with a repeated measures ANOVA, no effects of copper exposure, duration of exposure or copper*duration interactions were detected (Table 4.14).

Table 4.14: Repeated measures ANOVA of condition index of intertidal mussels exposed to 0 μg Cu L^{-1}, 100μg Cu L^{-1}, 250μg Cu L^{-1}, and 500μg Cu L^{-1} after 3, 7 and 14 days of exposure.

Source of Variation	SS	df	MS	F	p
Copper concentration	198	1	197.8	0.442	NS
Duration of exposure	1245	2	622.7	1.68	NS
Copper concentration*Duration of exposure	724	2	361.9	0.976	NS

The trend reflected in the repeated measures ANOVA started on day three when the condition index of the four groups of mussels were statistically similar (ANOVA $F_{(3,15)}$ = 1.28, p = 0.30) and remained similar for days seven (ANOVA $F_{(3,15)}$ = 1.16, p = 0.33) and fourteen (ANOVA $F_{(3,15)}$ = 1.60, p= 0.23).

4.4 Discussion

The first copper exposure experiment in this chapter showed no tendencies toward a dose dependent response to copper. As a result, experiments two and three were designed to expose mussels to acute concentrations of copper which would caused readily measurable physiological responses to copper. In these two experiments, the 500 μg Cu L^{-1} and 1,000 μg Cu L^{-1} treatments were lethal to the mussels but sublethal effects were still undetectable for the 100 μg Cu L^{-1} treatments. This lack of sublethal effects at 100 μg Cu L^{-1} was probably because clearance and respiration rates determined for the controls were low compared to rates determined for this species in previous research (Waite, 1989; Weatherhead, 1993). These low rates and the lack of significant treatment effects in experiments two and three may have been due to uncontrolled sources of variability. In the last experiment in this chapter, materials and methods were changed and resulted in an increase in the physiological rates recorded for the controls. In addition, significant differences between the controls and the 100 μg Cu L^{-1} treatments were detected (Table 4.13). Sublethal effects that were noted in experiment four included reduced clearance, respiration and SfG.

Results from the four experiments reported in this chapter showed that copper was lethal to *P. canaliculus* held at 15°C at concentrations of 250 μg Cu L^{-1} or greater. Complete

mortality which occurred nine days after exposure to concentrations of 250 μg Cu L^{-1} and 500 μg Cu L^{-1} in experiment four indicated that at these concentrations, copper acted as an irreversible toxic material and was not serving as an essential element. Experiment four also revealed sublethal effects of copper on intertidal mussels at a concentration of 100 μg Cu L^{-1}.

The sublethal effects of copper detected in the current experiments concur with previous research which have shown physiological effects of copper on bivalves from estuaries and waterways adjacent to industrial and urban centers in Europe (Munari and Mistri, 2007), North America (Schiff *et al.*, 2007) and Asia (Jeng *et al.*, 2000; Chiu *et al.*, 2000). Other sublethal effects may include changes in appearance of shellfish as in one case from Taiwan where discoloration of oysters during the "green oyster" incident of 1986 was due to anthropogenic copper pollution (Jeng *et al.*, 2000). Anthropogenic copper pollution also affected the macro invertebrate species richness and species number in freshwater environments (Sheehan and Winner, 1984) in North America. These actual cases of copper pollution show that the need for precaution was justified (Gee, 2006) only with the benefit of hindsight. This could also be true for locations such as Chile and Australia where copper pollution is less intense but is persistent (Warnken *et al.*, 2004; Lee and Correa, 2005).

At present, copper pollution is not known to be a serious problem in New Zealand although considerable amounts of this metal is used in the forestry industry as a lumber treatment and as a fungicide for control of *Dothistroma pini* which affects *Pinus radiata*, *P. nigra* subsp. *laricio*, and *P. ponderosa* (Bulman *et al.*, 2004). The quantity of copper used in prophylactic applications on pine plantations in New Zealand can be as high as 200 tonnes cuprous oxide per year (Bulman *et al.*, 2004) applied at a rate of 1.66 kg ha^{-1} of a dust which is between 45-50% copper. Other sources of copper in the New Zealand environment includes copper roofing, vehicle fluid leaks, vehicle break pad wear and antifoulant paints used on marine vessels. Combined, these sources could have contributed to increased copper levels in sediment and the waters of Wellington Harbour (Stoffers *et al.*, 1986) and the Waitemata Harbour in the 1980s (Hayward *et al.*, 2004). These detectable increases of copper in the aquatic environment in New Zealand highlight the need for biological assay techniques capable of detecting pollutants using the energetics of filter feeders such as *Perna canaliculus*. However, as was shown in the first three

experiments in this chapter, biological assays using live bivalves can easily yield inconsistent data. This is because issues such as handling, excitement, the metabolic status of the mussels and the operating procedures used are important to achieve statistically significant results. After a standard method was developed, experiment four was able to showed that clearance, respiration and SfG of *P. canaliculus* were significantly affected by 100 μg Cu L^{-1} and that clearance was the physiological index which was most sensitive to copper.

Clearance rate

Clearance assays measure rapid physiological responses in the bivalves (Toro *et al.*, 2003) but are very sensitive to factors such as age and size of the mussels and the temperature or particle concentration of the water (Sukhotin and Portner, 2001). These factors may have contributed to the clearance rates ranging between 1.6 L g^{-1} $h^{-1}$and 6.3 L g^{-1} h^{-1} for the control mussels between days one and three in the first experiment. The high values of 5.28 ± 1.13and 4.41 ± 1.57 L g^{-1} h^{-1} recorded on days three and seven were considerably above the range of 1.1-3.7 L g^{-1} h^{-1} determined for high and low shore level intertidal *P. canaliculus* sourced at Taylor's Mistake (Weatherhead, 1993). However, the range in the first copper exposure experiment included the clearance rate of 5.3 L g^{-1} h^{-1} which was determined for farm mussels (Waite, 1989) from the Marlborough region.

In experiments two and three, the clearance rates for the controls were low and no significant differences were detected between the controls and mussels exposed to 100 μg Cu L^{-1}. This suggested that factors which were not controlled by the experiment may have superseded the effects of copper. Previous studies have shown than the filtration rates of *Perna* mussels was sensitive to 160 μg Cu L^{-1} (Watling, 1981) and clam filtration was found to be very sensitive to 10 μg Cu L^{-1} (Munari and Mistri, 2007) so the current results were unexpected. Although effects of 100 μg Cu L^{-1} were not detected in the early experiments, results from eexperiments two and three showed that concentrations of 500 μg Cu L^{-1} and 1000 μg Cu L^{-1} were acutely toxic to both farm and intertidal *P. canaliculus*.

After experiments two and three had been completed, the exposure procedures were modified to ensure that the mussels would only have to be handled minimally when mussels were sampled for determination of clearance, respiration and excretion in

experiment four. This issue of handling of mussels had been addressed in previous methodologies used for *P. canaliculus* (Waite, 1989) and related species (Bruner *et al.*, 1994; Rajagopal *et al.*, 2005; Elliott *et al.*, 2008). Another improvement of the methodology was that clearance rate was determined using *Tetraselmis chuii*, the same alga used to feed the mussels each day for the duration of the experiment. This improvement in methodology resulted in an increased in filtration in experiment four. Whereas the clearance rates were 0.75 L g^{-1} h^{-1} and 1.89 L g^{-1} h^{-1} on day three for experiments two and three respectively, the clearance for the controls was 3.66 L g^{-1} h^{-1} on day three in experiment four. This rate increased modestly to 4.99 L g^{-1} h^{-1} on day fourteen in experiment four. Other studies have shown increases in clearance recorded for intertidal mussels brought to standard laboratory conditions for fifteen days (Labarta *et al.*, 1997). In addition to increasing over time, average clearance for the controls remained considerably higher than clearance for mussels exposed to 100 µg Cu L^{-1}. The significant difference between these treatments lasted for the duration of the experiment.

Respiration rate

The respiration rates in the first experiment were slightly lower than previously shown for *P. canaliculus* (Marsden and Weatherhead, 1998). The rates remained low for farm mussels in the second exposures. Respiration was variable in the last two experiments but were close to values from previous research on this species (Marsden and Shumway, 1993; James *et al.*, 2001).

No effect of copper was detected by repeated measures ANOVA conducted on respiration data in the first three experiments. However, in experiment four, exposure to copper resulted in a decrease in the respiration rate (Table 4.11). Specifically, the controls in this experiment respired at 0.18 ± 0.05 ml O_2 g^{-1} h^{-1} while the respiration rate for the 100 µg Cu L^{-1} exposed mussels was 0.09 ± 0.03 ml O_2 g^{-1} h^{-1}. (Figure 4.12). These were significant differences which resulted because respiration in the 100 µg Cu L^{-1} treatments declined over the fourteen days of the experiment (Table 4.11). Previous studies have shown that, mussel respiration rates may decrease as a result of valve closure, severe gill damage from metals, inhibition of respiratory enzymes or bradycardia caused by exposure to toxins (Anandraj *et al.*, 2002).

Excretion

Excretion was only measured for the last experiment conducted in this chapter. The rates determined in this experiment showed that the quantity of ammonia excreted by the controls and the 100 μg Cu L^{-1} treated mussels were consistent and similar for the three sampling days. While the rates in the 100 μg Cu L^{-1} treatment were stable, excretion in the 250 μg Cu L^{-1} and 500 μg Cu L^{-1} treatments were slightly elevated compared to the controls and the 100 μg Cu L^{-1} treatments on day 3. Also, the respiration rate for the 500 μg Cu L^{-1} treated mussels was significantly lower than the control on day three. This suggested that these copper exposed mussels had entered into anaerobic metabolism, relying to some degree on body reserves for maintenance (Cheung and Cheung, 1995).

This dynamic of elevated excretion and depressed respiration was not evident for the 250 μg Cu L^{-1} treated mussels because the respiration rate of these was never very low. This suggested that these mussels never reverted to anaerobic metabolism. Also, between days three and seven, mussels in the 250 μg Cu L^{-1} treatment underwent a slight decrease in excretion. This may have been linked to the reduction in clearance between days three and seven.

Scope for Growth

Scope for Growth was only determined for intertidal mussels used in the last experiment. The average SfG of 11.97 + 1.90 J g^{-1} h^{-1} was low compared to the 45.6 J g^{-1} h^{-1} and 613 J g^{-1} h^{-1} determine for intertidal *P. canaliculus* from the Cook Straight in winter (Gardner, 2000; Helson and Gardner, 2007). The values in the present study were higher than the –16.9 J g^{-1} h^{-1} and -20.2 J g^{-1} h^{-1} recorded for raft cultured and wild mussels which only had low levels of natural productivity on which to subsist (Gardner and Thompson, 2001).

The SfG determined in the present experiments was affected by copper at concentrations which could occur in copper polluted industrial zones (Sheehan and Winner, 1984; Ayres *et al.*, 2002; Krång and Ekerholm, 2006). This study can also be compared to a previous study on SfG of *P. canaliculus* which determined that mussels which had been transplanted from the pristine Mahanga Bay to sewage affected Fitzroy Bay attained higher SfG at Fitzroy Bay (Anderlini, 1992). On the other hand, the SfG of *Mytilus edulis* held in seston

poor mesocosms for an extended period displayed lower clearance and SfG compared to a field mussels (Widdows and Johnson, 1988).

The results from this research showed that SfG of mussels was depressed by copper at concentrations of 100 μg Cu L^{-1}. As a result, if mussels were deployed to an environment where background concentrations of copper was elevated, it is likely that the SfG would be depressed. Mussels could therefore be used as bioindicators in waters where the wildlife may be chronically exposed to levels of contaminants known to cause long term effects such as decreased fecundity (McDowell *et al.*, 1999). In our case, we did not look at the reproductive effort but we were able to show that at low levels of copper, the energetic balance of *P. canaliculus* was compromised. The reason why energetic balance was compromised was because SfG in mussels exposed to 100 μg Cu L^{-1} declined to less than 10% of SfG in the control mussels. This was because clearance rate decreased while respiration rate remained the same. This means that copper treated mussels had reverted to their body reserves to make up for lower food intake. Although the mussels in the 100 μg Cu L^{-1} seawater had survived, it is very likely that, in the medium or long term, these mussels would have died. These results show that the SfG methodology is very relevant where mussels are exposed to sub-lethal levels of inorganic copper.

Chapter 5 Effects of cadmium on scope for growth of farm and intertidal *Perna canaliculus*

5.1 Introduction

Sub-lethal effects of cadmium on wildlife such as bivalves are of concern to marine ecologists because background concentrations of this metal have increased considerably in the natural environment over the last fifty years (Nriagu, 1990; Bennet-Chambers *et al.*, 1997; Dietz *et al.*, 1998). Monitoring for this metal is not routinely conducted in most environmental protection laboratories (Burger, 2008) because precise quantification of cadmium requires an atomic absorption spectroscopy capability. Consequently, only a few studies have attempted to clarify whether recent increases of cadmium in the aquatic environment (Butler and Timperley, 1996) have resulted in physiological or pathological changes in aquatic species (Nicholson and Osborn, 1983; Kannan *et al.*, 2006).

Although research into the effects of cadmium on wildlife is now being developed, indications are that cadmium may have caused sublethal effects in humans and some animals at environmental concentrations previously believed to be harmless (Dietz *et al.*, 1998; Järup *et al.*, 2000). This suggests that monitoring for cadmium should be given higher priority by environmental protection agencies. This is especially relevant in coastal fishery resources which occupy waters affected by agricultural or industrialised watersheds (Butler and Timperley, 1996).

Examples of the importance of cadmium to bivalves include instances such as when internationally traded *Crassostrea gigas* oysters from British Columbia were rejected by the Hong Kong Food and Environmental Hygiene Department in 1999 and 2000, on the basis that the cadmium content in this seafood was in excess of Hong Kong's 2 $\mu g\ g^{-1}$ limit (Lekhi *et al.*, 2008). The source of cadmium in the British Columbia oysters was believed to be naturally occurring dissolved cadmium (Lekhi *et al.*, 2008). The aquaculture sector in British Columbia has since recognised that sea based farms should be located at sites where cadmium levels were low. This issue of shellfish with elevated levels of cadmium is important because the oyster and scallop industries in British Columbia (Kruzynski, 2004) have become threatened by non-tariff trade barriers imposed.

Previous research also suggest that the dynamics of cadmium in seawater in southern New Zealand and British Columbia may be similar because in both cases, the cadmium was from natural sources. In the case in British Columbia, *Crassostrea gigas* accumulated 2.63 mg Cd g^{-1} wet weight while the oysters from the Foveaux Strait have accumulated as much as 9 mg Cd g^{-1} wet weight (Frew *et al.*, 1997). Direct analysis of seawater in the Foveaux Strait showed that the cadmium concentration in the water was low (Croot and Hunter, 1998). This is believed to be because cadmium may be assimilated by zinc limited plankton which undergo subsidence into the benthos. In the benthic environment, the phytoplankton may be consumed by filter feeders such as Foveaux Strait dredge oysters *Tiostrea chilensis* which have accumulate Cd at levels exceeding 9 $\mu g\ mg^{-1}$ (Nielsen, 1975).

Although there is much concern regarding the toxicity of cadmium in the aquatic environment, only a few studies have determined the effects of cadmium on the physiology of bioindicators such as bivalves. Some of the existing literature suggest that non essential elements like cadmium are more toxic than essential trace elements such as copper or zinc (Eisler, 1986; Islam and Tanaka, 2004; Giarratano *et al.*, 2007). However, other sources suggests that cadmium is less toxic to bivalve filter feeders than metals such as mercury, copper and tributyltin (Gosling, 2003; Mubiana and Blust, 2007). As a result, bivalves such as *Lamellidens marginalis* have been considered for use as biofilters in water treatment facilities specifically for the purpose of sequestration of the dissolved cadmium load (Jana and Das, 1997). This application is based on the fact that some bivalves have

been known to survive with a burden of 13 mg Cd kg fresh weight^{-1}, a concentration which would be acutely toxic to humans (Eisler, 1985).

Although bivalves like the British Columbia oysters accumulated very high levels of cadmium, few studies have shown physiological effects of this metal on marine invertebrates. For example, no correlation was found between the tissue concentration of cadmium and the scope for growth (SfG) of the cockle *Anadara trapezia* which were transplanted from unpolluted to polluted sites in New South Wales (Burt *et al.*, 2007). In addition to bivalves, the SfG of other marine species such as *Callinectes* crabs was not affected by 100 μg Cd L^{-1} at 25 ppt (Guerin and Stickle, 1999). However, the SfG of the gastropod *Nassarius festivus* was shown to be sensitive to cadmium at 160 μg L^{-1} and SfG was a more sensitive biomarker for exposure to cadmium than RNA/DNA, shell length and tissue weight (Wo *et al.*, 1999). Few publications have described how the SfG of New Zealand green lipped mussels would be affected by concentrations of cadmium similar to the concentrations tested above. Elevated concentrations of cadmium in marine bivalves are thought to be related to agricultural fertilizer use in Mahurangi Harbour (Butler and Timperley, 1996; Perera, 2004).

The experiments in this chapter were therefore designed to determine if cadmium in seawater caused changes in physiological rates measured on intertidal and farm *P. canaliculus* mussels. In the first experiment, concentrations of 500 μg Cd L^{-1}, 1,000 μg Cd L^{-1} and 1,500 μg Cd L^{-1} were used to determine if these concentrations were lethal to mussels. In the second experiment, intertidal mussels were exposed to 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1}. The third experiment used farm mussels exposed to these same concentrations of cadmium. Farm and field mussels were compared to determine whether these groups showed any differences in physiological responses to cadmium. The cadmium concentrations were chosen because they were within the order of magnitude of 40 μg Cd L^{-1} which has been identified as the acute value of cadmium which should not be exceeded in a twenty-four hour period more than once every three years (USEPA, 2001).

5.2 Methods

List of experiments

The three experiments in this chapter tested the effects of cadmium on farm and intertidal mussels:

Experiment 1: Effects of 0 μg Cd L^{-1}, 500 μg Cd L^{-1}, 1,000 μg Cd L^{-1} and 1,500 μg Cd L^{-1} on intertidal mussels.

Experiment 2: Effects of 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66μg Cd L^{-1} and 99μg Cd L^{-1} on intertidal mussels.

Experiment 3: Effects of 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66μg Cd L^{-1} and 99μg Cd L^{-1} on farm mussels.

Experiment 1: Effects of 0 μg Cd L^{-1}, 500 μg Cd L^{-1}, 1,000 μg Cd L^{-1} and 1,500 μg Cd L^{-1} on intertidal mussels (July-August 2007).

Collection and maintenance

Mussels were collected at Taylors Mistake on July 13, 2007 and transported to the SBS aquarium where they were prepared for the experiment on the day after collection. Preparation involved removing barnacles with a knife and removing epiphytic algae before attaching each mussel to a plastic plate. The mussels were then suspended in slow running seawater to ensure that residues of the cyanoacrylate adhesive would be removed before starting of the experiment. During this time, mussels were fed once per day with concentrated frozen *Tetraselmis chuii*. This time was also used to fill the exposure system with seawater and to adjust aeration. Cadmium stock solutions were also made during this time.

Experimental design

This experiment was designed to determine the effect of 0 μg Cd L^{-1}, 500 μg Cd L^{-1}, 1,000 μg Cd L^{-1} and 1,500 μg Cd L^{-1} on twenty mussels held at 15°C for fourteen days. On the

day when the experiment was started, all twenty mussels per treatment were stocked together.

Experimental setup

The exposure system used in this experiment was the flow-through system described in section 2.5. The twenty attached mussels were stocked in exposure tanks filled with seawater to begin the experiment. At the start of the experiment, the seawater was spiked with the appropriate aliquot of a stock solution of cadmium in the header and exposure tanks. The stock solution was made by adding 11.27 g $CdCl_2 \cdot 2\frac{1}{2}H_20$ to 100 ml distilled water. To attain concentrations of 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}, 333 µl, 666 µl and 1,000 µl of the stock were applied to tanks two, three and four. Mussels were then fed with concentrated *T. chuii* each day and the tap controlling gravity flow from the spiked header tank was turned on and water exchange was commenced. On the following day, water which had been exchanged by gravity flow was removed and discarded. New seawater was added to the header tank and spiked with the cadmium stock each day.

Mussels were checked for mortality each morning after the first day of exposure. Checking was done between 6:00 and 7:00 AM by lifting the plates on which the mussels were attached (Figure 2.8) and observing for changes in the gaping of individual mussels. If mussels did not close their valves, they were suspected to be dead. When this was observed, the entire group of 4 or 5 mussels was removed from the tank and each mussel was checked for slow movement of the valves on exposure to air. If slow movement of valves was noted, mussels were replaced into the tanks from which they were taken. If mussels were dead, they were discarded.

Because no mortality had occurred, clearance, respiration, excretion and condition indices were measured for n = 8 mussels from the four treatments after fourteen and fifteen days of exposure. Clearance was measured at 9:00 - 11:00 AM followed by two batches of combined respiration and excretion measurements starting at 1:00 PM. At the end of both days, the sixteen mussels on which physiological measurement were conducted were shucked and the soft tissue separated from the shell. Both parts were placed to dry at 55°C

for two days and the dry weight data used to calculate the physiological indices on a dry weight basis. These weights were also used to calculate the condition indices and clearance, respiration and excretion rates on a dry weight basis.

Experiment 2: Effects of 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1} on intertidal mussels (February 2007).

Collection and maintenance

Two hundred mussels in the 5.9 cm -7 cm size class were collected from Taylor's Mistake on February 21, 2007. These mussels were prepared for the experiment by first removing all barnacles and epibionts. The mussels were then attached to individual plastic plates previously described. Mussels were maintained in the SBS aquarium at 13°C and provided with feed until the experiment was started on February 25.

Experimental design

In this fourteen day experiment, a total of ninety six attached intertidal *P. canaliculus* mussels were subject to four exposure treatments of 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1} and were evaluated for physiological responses. In each of the treatments, 24 attached mussels were stocked over two days to allow a sample of n = 8 mussels to be collected after three, seven and fourteen days of exposure. Stocking of the experiment was staggered over two days to allow for sampling of n = 4 mussels for each treatment on one day. The other n = 4 mussels were sampled on the subsequent day.

Experimental setup

The exposure system used for this experiment has previously been described in Section 2.5. The four exposure treatments were applied to the four exposure tanks by pipetting 333 μl, 666 μl and 999 μl of a stock made from 7.51 g of $CdCl_2 \cdot 2½H_20$ in 1,000 ml distilled water into each header tank. On each day, new seawater was placed in the header tanks and allowed to flow into the exposure tanks at a rate of between 25-30 ml $minute^{-1}$. This

allowed for exchange of the water in each exposure tank to occur over 20 hours. Each day, the mussels were fed and checked for mortality.

Evaluation

After three, seven and fourteen days of exposure, two groups of n=4 mussels were removed from each experimental tank according to the staggered stocking schedule previously described. The mussels which were sampled were evaluated for clearance, respiration and excretion using the methods described in Sections 2.6 and 2.7. After each day of the physiological evaluations, the mussels were shucked and the tissues and shells separated and dried. The physiological rates were then calculated and analyzed as described in Section 2.10.

Experiment 3: Effects of 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66μg Cd L^{-1} and 99μg Cd L^{-1} on farm mussels. (March 2007).

Collection and maintenance

Farm mussels which had been sourced from Pigeon Bay on March 19, 2007 were held temporarily at the SBS aquarium and prepared for the exposure experiment. The mussels were cleaned of encrustations or epibionts and each mussel secured to a perspex plate using cyanoacrylate glue. This ensured that the mussels would secrete byssus unto the plates before starting of the experiment. Mussels were then kept in slowly running seawater to remove traces of the cyanoacrylate before transfer to exposure systems at the start of the experiment

Experimental design

For this experiment, twenty four mussels were stocked in each tank and exposed to seawater spiked with 0 μg Cd L^{-1}, 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1}. These twenty four mussels were stocked over two days to allow for measurements of clearance,

respiration, excretion and condition to be conducted on four mussels per treatment per day. This allowed evaluation of a sample size of n = 8 mussels over two days.

Experimental setup

The experiment was started on 22 March, 2007 utilizing the exposure system described in Section 2.5. The four exposure treatments were applied using one 1,000 µl pipette to apply 333 µl, 666 µl and 999 µl of a stock made from 7.51 g of $CdCl_2 \cdot 2½H_20$ in 1,000 ml distilled water into the headers for exposure tanks two, three and four. Every morning, each tank was checked for mussel mortality and new seawater was placed in the header tanks then spiked. After three, seven and fourteen days of exposure to the cadmium spiked seawater, n = 8 mussels were sampled over two days and evaluated for clearance, respiration and excretion rates as well as condition.

Evaluation

After all physiological data for n =8 mussels were collected for the three time intervals, calculations of clearance, respiration and excretion were made on a dry weight basis as in Sections 2.6 and 2.7. The data were then analysed and presented as in Section 2.10.

5.3 Results

Experiment 1: Effects of 0, 500, 1,000 and 1,500 µg Cd L^{-1} on intertidal mussels (August 2007).

The results from this experiment were that concentrations 1,500 µg Cd L^{-1} did not cause mortality to intertidal *P. canaliculus* but that between 500 - 1,000, µg Cd L^{-1} the physiology of *P. canaliculus* was affected.

Mortality and clearance

There was no mortality of mussels in any of the treatments over the fourteen days of exposure. Clearance, respiration and excretion were then determined and the SfG and condition indices calculated for these mussels.

After fourteen days of exposure, the clearance rates determined for mussels exposed to the cadmium showed declining clearance with increasing concentration of exposure. Clearance ranged from 4.58 ± 0.38 L g^{-1} hr^{-1} for the controls to 1.75 ± 0.24 L g^{-1} hr^{-1} for the 1,500 µg Cd L^{-1} treatments. The decrease in clearance between the 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1} treatments was not significant (Figure 5.1).

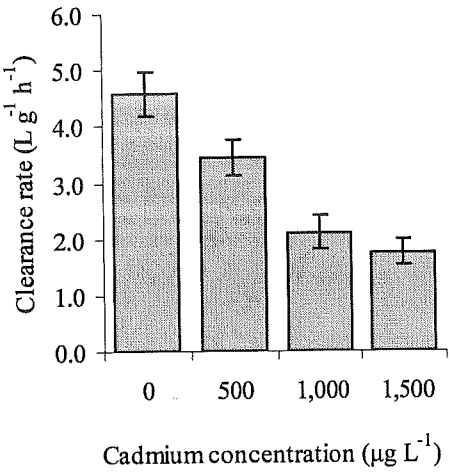


Figure 5.1: Clearance rate +/- SE of intertidal *P. canaliculus* after 14 days of exposure to 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

The differences among the four treatments were found to be statistically significant (ANOVA $F_{(3,31)} = 16.60$, $p<0.001$). *Post hoc* evaluation using Tukey's HSD test showed that clearance rates for the control and the 500 µg Cd L^{-1} treatments were similar but different from the other group comprising the 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1} treatments.

Respiration

Respiration rates for the four treatments ranged between 0.14 ± 0.02ml O_2 g^{-1} h^{-1} for the controls and 0.10 ± 0.02 ml O_2 g^{-1} h^{-1} for all other treatments (Fig 5.2).

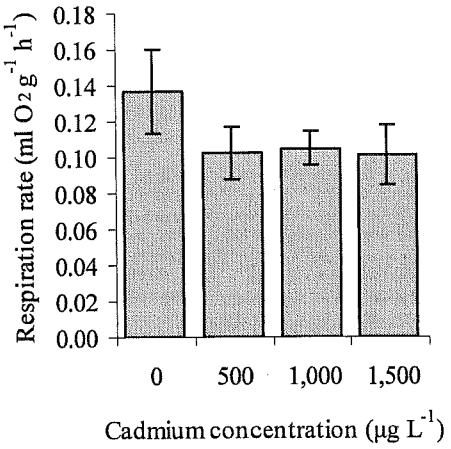


Figure 5.2: Respiration rate +/- SE of intertidal *P. canaliculus* after 14 days of exposure to 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

Analysis of variance of these data showed no statistically significant differences among these treatments (ANOVA $F_{(3,31)} = 1.06$, $p = 0.38$).

Excretion

Excretion data generated at the end of the fourteen day period showed that the mean rate of excretion in the controls was 48.2 ± 12.64 µg NH_4^+ g^{-1} hr^{-1} while the 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1} treatments recorded excretion rates of 25.31 ± 8.66 µg NH_4^+ g^{-1} hr^{-1}, 52.52 ± 13.20 µg NH_4^+ g^{-1} hr^{-1} and 74.01 ± 32.04 µg NH_4^+ g^{-1} hr^{-1} respectively. These values were variable, showing a numeric increase with increasing concentration of cadmium (Fig 5.3). However, when the data were analyzed with ANOVA, no significant differences were detected (ANOVA $F_{(3,31)} = 1.11$, $p = 0.36$).

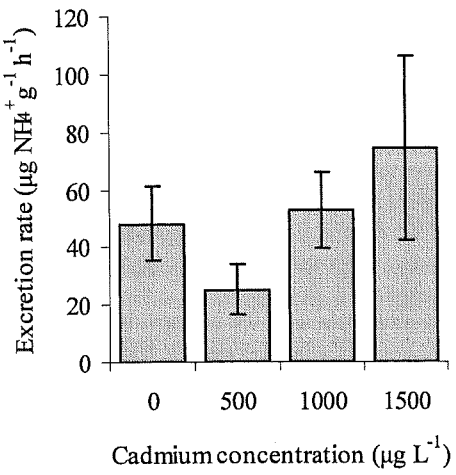


Figure 5.3: Excretion rate +/- SE of intertidal *P. canaliculus* after 14 days of exposure to 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

SfG

Average SfG in this experiment declined with increasing concentration of cadmium (Figure 5.4).

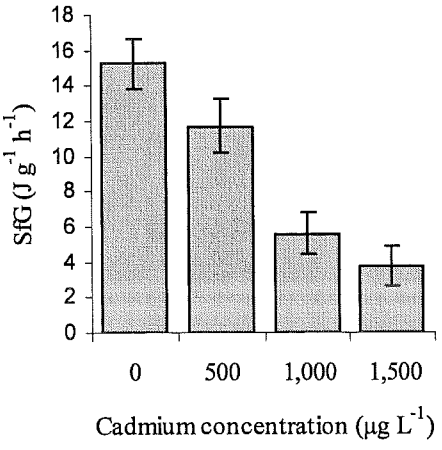


Figure 5.4: SfG +/- SE of intertidal *P. canaliculus* after 14 days of exposure to 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

After fourteen days, differences in scope for growth were detected based on the concentration of cadmium (ANOVA $F_{(3,31)} = 16.02$, $p<0.001$). The average rates were 15.24 ± 1.38 J g^{-1} hr^{-1}, 11.67 ± 1.53 J g^{-1} hr^{-1}, 5.59 ± 1.22 J g^{-1} hr^{-1} and 3.76 ± 1.15 J g^{-1} hr^{-1} for the 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1} treated mussels. *Post hoc* evaluation of the rates showed that SfG of the controls were not significantly different from the 500 µg Cd L^{-1} treatments but that the 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1} exposed mussels had lower SfG than the control mussels. This means that SfG of intertidal mussels was affected at a concentration between 500 µg Cd L^{-1} and 1,000 µg Cd L^{-1}.

Condition

After 14 days of exposure, condition in the four treatments ranged from a 65.80 ± 2.88 for the 1,500 µg Cd L^{-1} treatments to 75.45 ± 2.73 for the 500 µg Cd L^{-1} treatment (Figure 5.5).

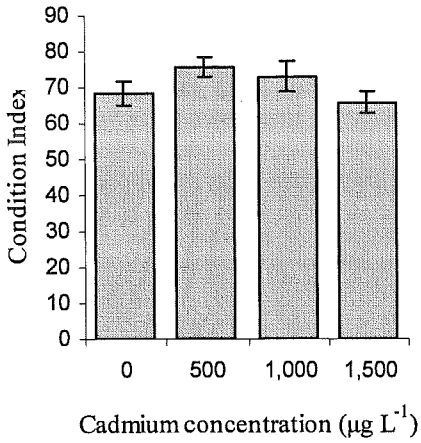


Figure 5.5: Condition index +/- SE of intertidal *P. canaliculus* after 14 days of exposure to 0 µg Cd L^{-1}, 500 µg Cd L^{-1}, 1,000 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

The values in this range were very similar, and, when compared using ANOVA, no significant differences were detected (ANOVA $F_{(3,31)}= 1.76$, $p = 0.17$). This shows that when exposed to highly contaminate seawater, the condition of *P. canaliculus* mussels did not decline within a time frame of fourteen days.

Experiment 2: Effects of 0, 33, 66 and 99 µg Cd L^{-1} on intertidal mussels (February 2007).

The results compiled in this section show that no effects of cadmium concentrations were detected for any of the physiological indices or condition index measured for the intertidal mussels tested. Significant effects of duration of the exposure were however detected for the clearance, excretion, SfG and condition index and are reported in more detail below.

Clearance rate

The data compiled on clearance by intertidal mussels showed that the rates calculated for the four treatments were comparable at each time interval (Figure 5.6). However, differences were evident between the averages recorded for day seven compared to the averages on days three and fourteen (Fig 5.6).

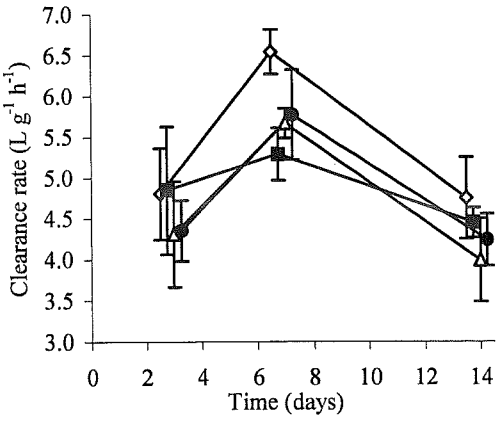


Figure 5.6: Clearance rate +/- SE of intertidal *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

Analysis of the dataset incorporating the results of days three, seven and fourteen using repeated measures ANOVA showed that there was no effect of concentration of cadmium on clearance (Table 5.1). However, there was a significant effect of duration of the exposure. The interaction of cadmium concentration and duration of exposures was not significant over the experiment.

Table 5.1: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on clearance rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	6.49	3	2.16	1.39	NS
Duration of exposure	39.04	2	19.52	11.15	<0.001
Cadmium concentration*Duration of exposure	4.19	6	0.70	0.40	NS

The significant effect of time was investigated with a *post hoc* Tukeys HSD test which showed that clearance rate on day seven was significantly greater than clearance on days three and day fourteen. In summary, concentrations of cadmium as high as 99 μg Cd L^{-1} did not affect the clearance rate of intertidal *P. canaliculus*.

Respiration rate

The data compiled for respiration rates of the four treatments on the three days showed that the averages for each treatment were generally similar throughout the experiment although a low value was recorded for the controls on day seven (Figure 5.7).

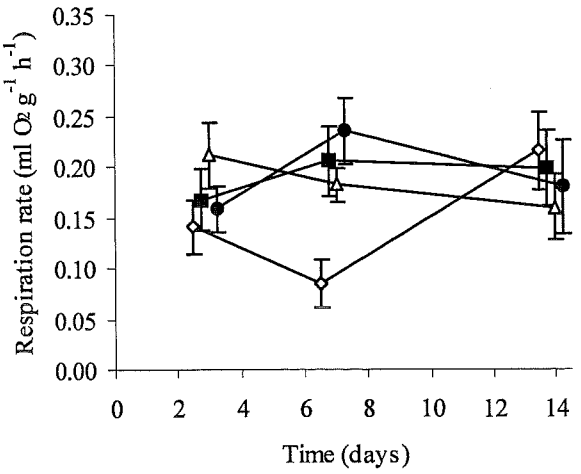


Figure 5.7: Respiration rate +/- SE of intertidal *P. canaliculus* exposed to 0 μg Cd L^{-1}(◇), 33 μg Cd L^{-1}(■), 66 μg Cd L^{-1}(△) and 99 μg Cd L^{-1}(●) at 34 ppt salinity on days three seven and fourteen.

Analysis of the data for the three time intervals using repeated measures ANOVA showed that no significant effects of concentration of cadmium or duration of exposure were detected over the course of the experiment but that the interaction between these was

significant (Table 5.2). The interaction was because the controls and the 99 µg Cd L^{-1} treatments which had been similar on day three diverged on day seven but converged again on day fourteen.

Table 5.2: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on respiration rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	<0.001	3	<0.001	2.37	NS
Duration of exposure	<0.001	2	<0.001	0.42	NS
Cadmium concentration*Duration of exposure	<0.001	6	<0.001	2.96	0.014

In summary, these data indicate that the concentrations of cadmium tested in this experiment (0-99 µg Cd L^{-1}) did not cause significant changes in the aerobic respiration rates of intertidal *P. canaliculus*.

Excretion rate

There was a high excretion rate for the controls on day three but this high rate declined steadily on days seven and fourteen (Figure 5.8).

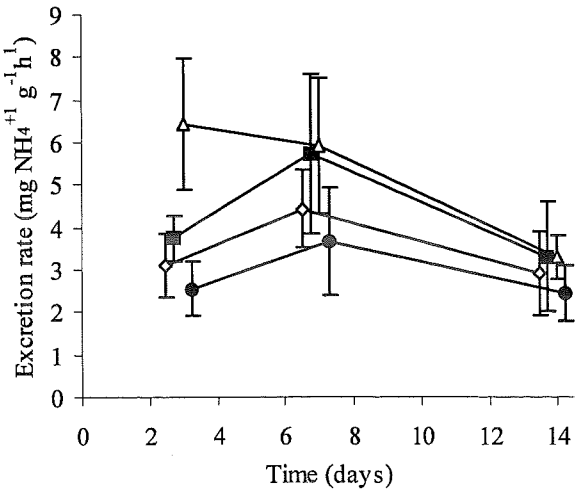


Figure 5.8: Excretion rate +/- SE of intertidal *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1} (■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

Analysis of the full data with repeated measures ANOVA also showed that there were no differences in excretion due to concentration of cadmium (Table 5.3) but that an effect of time was detected. *Post hoc* evaluation of the results from this test showed that the significant effect of time was because of the decline in excretion from 70.99 ± 15.47 µg NH_4^+ g^{-1} hr^{-1} on day three to 59.04 ± 14.04 µg NH_4^+ g^{-1} hr^{-1} on day fourteen.

Table 5.3: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on excretion rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	0.09	3	0.03	1.85	NS
Duration of exposure	0.14	2	0.07	4.19	0.020
Cadmium concentration*Duration of exposure	0.05	6	0.01	0.46	NS

Scope for growth

The data compiled for the intertidal mussels exposed to 0 µg Cd L^{-1}, 33 µg Cd L^{-1}, 66 µg Cd L^{-1} and 99 µg Cd L^{-1} for fourteen days showed that the values were similar for three of the treatments on the three days but that SfG of the controls was high on day seven (Fig 5.9).

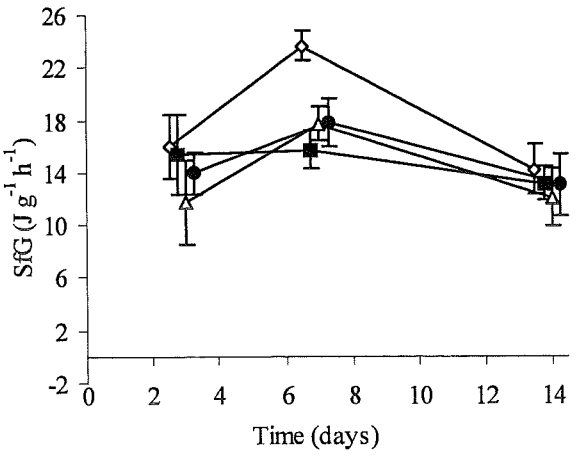


Figure 5.9: SfG +/- SE of intertidal *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1} (△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

When the data for the three time intervals were analyzed with repeated measures ANOVA, no effect of cadmium concentration was detected (Table 5.4). However, the repeated measures ANOVA showed significant differences in the average SfG recorded on the different days (Table 5.4).

Table 5.4: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on SfG of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	0.20	3	0.07	2.50	NS
Duration of exposure	0.49	2	0.25	6.63	0.003
Cadmium concentration*Duration of exposure	0.13	6	0.02	0.57	NS

The *post hoc* Tukeys HSD test showed this was because SfG increased from 15.9 $\pm$ 2.37 J g^{-1} hr^{-1} on day three to 23.60 $\pm$ 1.14 J g^{-1} hr^{-1} on day seven.

Condition index

There was a gradual decrease in condition with time and considerably more variability in the data on day fourteen compared to day three and day seven (Figure 5.10).

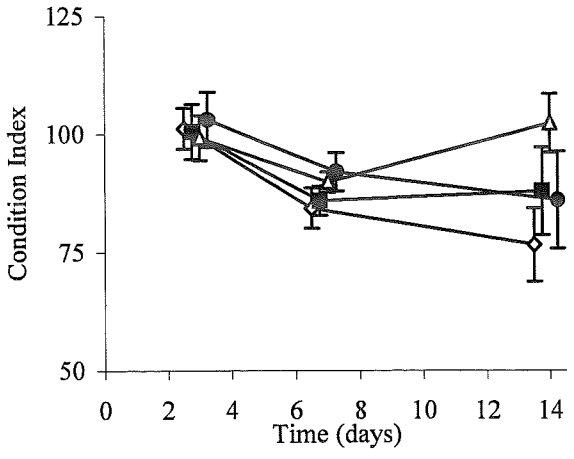


Figure 5.10: Condition index +/- SE of intertidal *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

Also, the data showed that most treatments were similar on day three when no effects of exposure to cadmium were evident. Although there was a decrease in condition on day seven, no effects of cadmium were evident. The results of the repeated measures ANOVA showed that while cadmium concentration did not affect condition, the effect of duration was significant (Table 5.5).

Table 5.5: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on condition index of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	0.116	3	0.039	2.058	NS
Duration of exposure	0.308	2	0.154	6.029	0.004
Cadmium concentration*Duration of exposure	0.174	6	0.029	1.134	NS

Post hoc analysis of the data using Tukeys HSD test confirmed that the condition of 100.9 $\pm$ 5.20 recorded on day three was significantly greater than the 88.14 $\pm$ 8.35 recorded on day fourteen.

Experiment 3: Effects of 0, 33, 66 and 99 µg Cd L^{-1} on farm mussels (March 2007).

Results compiled for this experiment showed that no effects of cadmium were detected at any of the concentrations tested. However, clearance and SfG was shown to increase significantly over time. Also, mussels in the control treatments spawned on the second day of the experiment.

Clearance rate

Clearance rates determined for farm mussels in this experiment were considerably lower (Figure 5.11) than the clearance rate determined for intertidal mussels in the pervious experiment (Figure 5.6).

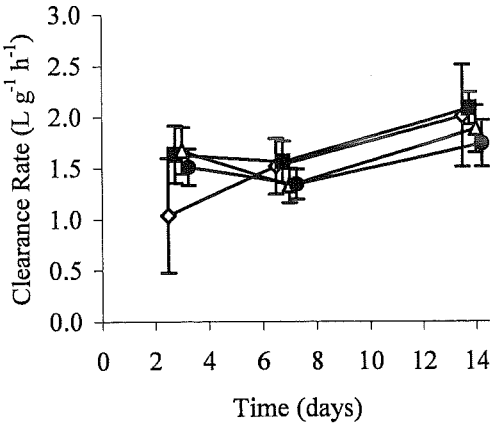


Figure 5.11: Clearance rate +/- SE of farm *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

Clearance rates for the farm mussels in this experiment showed a gradual rise between days three and fourteen. This increase was considerably higher for the controls which doubled their clearance from 1.04 ± 0.18 L g^{-1} hr^{-1} to 2.01 ± 0.29 L g^{-1} hr^{-1} between days three and fourteen (Figure 5.11).

The different cadmium exposures did not result in significantly different clearance rates (Table 5.6). An effect of the duration of the exposure was however detected by the repeated measures ANOVA procedure indicating that the rise in clearance over the 14 days was significant.

Table 5.6: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on clearance rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	0.89	3	0.30	0.70	NS
Duration of exposure	4.92	2	2.46	8.22	0.001
Cadmium concentration*Duration of exposure	2.05	6	0.34	1.14	NS

Post hoc comparisons using Tukey's HSD test confirmed that the effect of duration of exposure was because the average clearance on days three and seven were lower than clearance on day fourteen.

Respiration rate

The respiration rates determine for the four treatments over the three sampling days showed similar rates among the treatments at each interval and similar rates between the intervals (Figure 5.12).

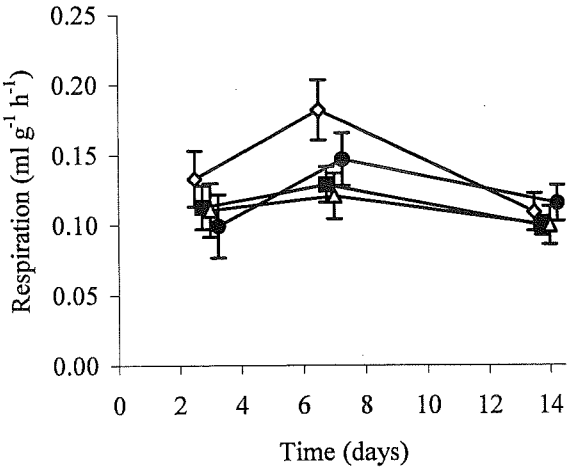


Figure 5.12: Respiration rate +/- SE of farm *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity on days three seven and fourteen.

Repeated measures ANOVA showed no effect of cadmium on respiration but there was an effect of duration of exposure (Table 5.7). *Post hoc* analysis of the significant differences using Tukey's HSD test showed that significant differences were noted between the respiration rates on days seven and fourteen.

Table 5.7: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on respiration rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	<0.001	3	<0.001	1.80	NS
Duration of exposure	<0.001	2	<0.001	6.50	0.003
Cadmium concentration*Duration of exposure	<0.001	6	<0.001	0.82	NS

Excretion rate

The excretion rates determined in this experiment displayed considerable variability on the three sampling days. On each sampling, excretion for the controls mussels was higher than for the other three treatments (Figure 5.13). On day 14, extremely high excretion was noted for a single mussel in the control tank.

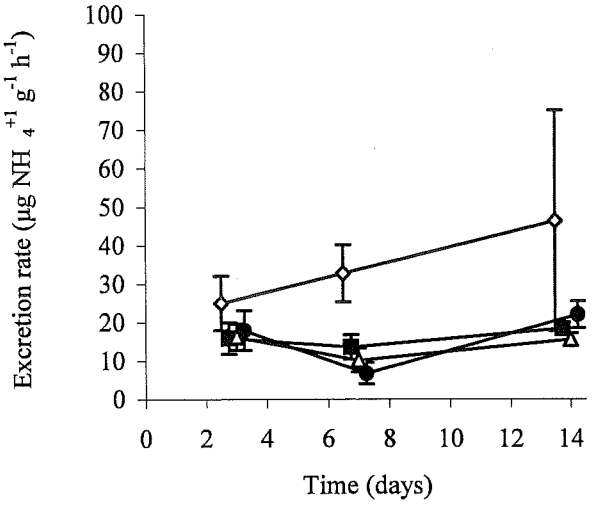


Figure 5.13: Excretion rate +/- SE of farm *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34ppt on days three seven and fourteen.

There was no significant effect of concentration of cadmium on excretion rates (Repeated Measures ANOVA, Table 5.8). There were no significant effects of exposure time or interactions between the duration and concentration of the exposures. However, over the

duration of the experiment, excretion in the controls was consistently higher than for other treatments and standard errors were high.

Table 5.8: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on excretion rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	0.03	3	0.0100	2.25	NS
Duration of exposure	0.01	2	0.0060	1.97	NS
Cadmium concentration*Duration of exposure	0.01	6	0.0010	0.30	NS

Scope for Growth

SfG of the controls was initially very low but this increased steadily over the period between days three and fourteen (Figure 5.14). The SfG of the other treatments showed only modest increases within this time.

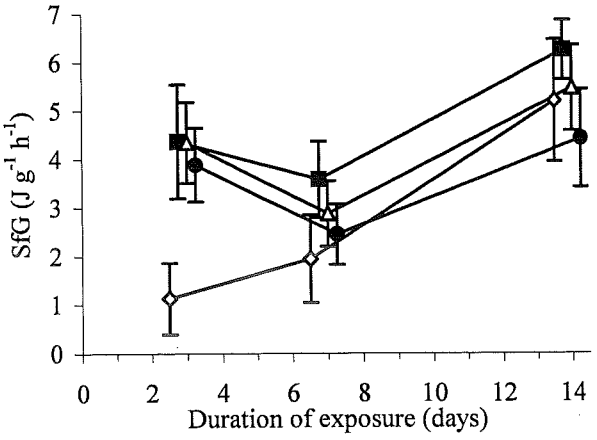


Figure 5.14: SfG +/- SE of farm *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1} (■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34 ppt salinity.

Analysis of all the data using repeated measures ANOVA showed that the SfG for the four cadmium treatments were similar over the duration of the experiment (Table 5.9). However, an effect of duration of exposure was detected.

Table 5.9: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on SfG of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	52.10	3	17.36	2.16	NS
Duration of exposure	119.00	2	59.49	11.26	<0.001
Cadmium concentration*Duration of exposure	32.00	6	5.33	1.01	NS

Post hoc evaluation with Tukey's HSD test showed that the significant effect of time emerged because the SfG recorded on day three and seven was significantly lower than the rates determined on day fourteen.

Condition Index

The condition of all mussels in the treatments were similar on day three as well as the subsequent days (Fig 5.15). A high average condition was noted for 33 µg Cd L^{-1} treatment on day three but this was due to one mussel which recorded a very high shell weight.

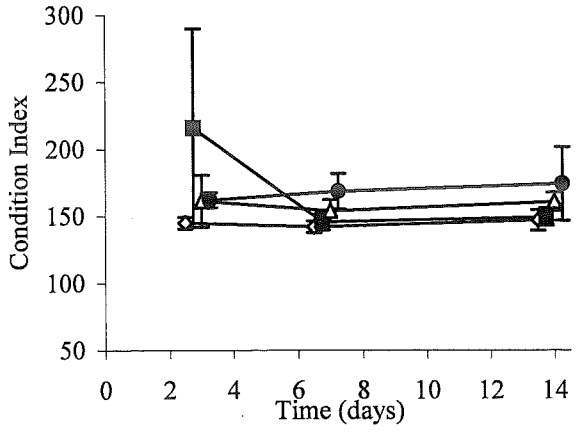


Figure 5.15: Condition index +/- SE of farm *P. canaliculus* exposed to 0 µg Cd L^{-1} (◇), 33 µg Cd L^{-1}(■), 66 µg Cd L^{-1}(△) and 99 µg Cd L^{-1} (●) at 34ppt on days three seven and fourteen.

Analysis of all the data by repeated measures ANOVA also showed that there were no significant effects caused by the concentration of cadmium exposure or the duration of the exposure (Table 5.10).

Table 5.10: Repeated measures ANOVA of effect of cadmium concentration and duration of exposure on condition index of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Cadmium concentration	9798.00	3	3266.00	0.65	NS
Duration of exposure	5791.00	2	2895.00	0.68	NS
Cadmium concentration*Duration of exposure	20000.00	6	3332.00	0.78	NS

5.4 Discussion

There are only a few publications which have described the sub-lethal effects of cadmium on bivalve filter feeders such as *Perna canaliculus* at latitudes of 45° or greater. This may be because there are only few bivalve fisheries or aquaculture facilities which can be directly affected by industrial discharge of cadmium at these latitudes. Although only a few studies have related bivalve physiology to cadmium content of seawaters in the high latitudes (Kruzynski, 2000), research areas are emerging which suggest that cadmium intoxications have occurred in marine mammals and seabirds at high latitudes (Muirhead and Furness, 1988; Lock *et al.*, 1992; Dietz *et al.*, 1998; Endo *et al.*, 2008; Rosa *et al.*, 2008).

The results from three experiments in this chapter showed that *P. canaliculus* is not affected by cadmium concentrations ranging from 33 μg Cd L^{-1} to 500 μg Cd L^{-1}. The onset of physiological effects of cadmium in *Perna canaliculus* only at high concentrations starting at between 500 μg Cd L^{-1} and 1,000 μg Cd L^{-1} was an unexpected result. *Perna canaliculus* is considered to be a sensitive species which is known to occur in relatively pollution free waters (Dawber, 2003). In the previous chapter, *P. canaliculus* showed highly significant physiological effects due to 100 μg Cu L^{-1}. It therefore seems that *Perna canaliculus* mussels may have some capacity to resist the toxic effects of cadmium or avoid contact with cadmium dissolved in seawater. This may be related to the low

temperatures at high latitudes because the slow growth rate at high latitudes may protect marine species from the toxic effects of cadmium and other contaminants (Dietz *et al.*, 1998). Another key finding from these experiments was that farm mussels displayed overall lower physiological and metabolic rates but considerably higher condition than intertidal mussels. The former result may be because farm mussels are considerably more sensitive to artificial laboratory conditions than intertidal mussels.

Acute (0 Cd L^{-1} - 1,500 Cd L^{-1}) exposure

In the first experiment, *Perna canaliculus* showed physiological effects of exposure at concentrations between 500 μg Cd L^{-1} - 1,000 μg Cd L^{-1}. The sub-lethal effects of the high levels of cadmium used in this experiment were most clearly shown by the clearance rate and SfG.

The physiological effects of cadmium on excretion were not as clear because of high excretion of the controls compared to the 500 Cd L^{-1} treatment. There could have been multiple sources of high excretion such as digestion of feed or de-amination of body reserves for maintenance. The lack of significant effects of cadmium on excretion in this experiment was probably because excretion rates by controls was high due to spawning.

The increase in excretion rate recorded between 500 μg Cd L^{-1} and 1,500 μg Cd L^{-1} could have reflected a tendency of increasing reliance on body reserves for maintenance, a physiological adaptation seen for *Mytilus edulis* (Widdows, 1978a). This may have been a result of valve closure by mussels which were attempting to avoid cadmium (El-Shenawy, 2004). A suggestion that valve closure occurred was the slightly lower respiration rates in the cadmium exposed mussels compared to the control (Figure 5.2). Also, the slightly lower respiration rate of the different treatments compared to the controls indicated that there was very little energy being allocated for detoxification. This may be because the mussels relied on a non-oxidative mechanism to cope with high levels of cadmium. On the other hand, increasing excretion with increasing cadmium concentration could also indicated an attempt to decrease the cadmium burden through elimination by increased urine production.

The exact mechanism which *P. canaliculus* utilizes to protect itself from cadmium is unknown at present. What is known is that the mechanism confers a high threshold of cadmium tolerance. Such high tolerance has previously been noted for *Mytilus edulis* (Eisler, 1985) and *Perna viridis* (Cheung and Cheung, 1995). In one previous study, only at concentrations of 7,500-10,000 μg Cd L^{-1} did cadmium affect the physiology of *Mytilus edulis* (Redpath and Davenport, 1998).

Sub-lethal (0 Cd L^{-1} - 99 Cd L^{-1}) exposure

Intertidal mussels

When intertidal mussels were exposed to cadmium at concentrations of 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1}, no effects on clearance, respiration, excretion or SfG were detected. For clearance, excretion and SfG however, an effect of time was detected. These effects of time may have originated from increased feeding between days three and seven. The cumulative effect was that for the intertidal mussels, the average SfG for the experiment was 17.91 J g^{-1} hr^{-1} for the controls and 14.74 J g^{-1} hr^{-1}, 13.83 J g^{-1} hr^{-1} and 14.91 J g^{-1} hr^{-1} for the 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1} treatments respectively. These values for SfG were statistically similar for the duration of the experiment. Finally, the overall decline in condition of the intertidal mussels suggest that all four treatments lost tissue due to a factor which affected all treatments in this experiment.

Farm mussels

No significant effects of 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1} were detected on any of the indices measured on the farm mussels. However, an effect of the duration of the exposure was detected for clearance, respiration and scope for growth. In terms of clearance, the rate measured for the controls was the lowest among the treatments on day three. The average for the controls then increased to the second highest rate by day fourteen. This contributed to a significant effect of time which was detected by repeated measures ANOVA.

All four groups of farm mussels showed increased respiration on day seven resulting in the significant effect of duration of exposure which was detected (Table 5.7). The experimental average SfG for the controls was the 2.76 J g^{-1} hr^{-1} which was the lowest SfG of all groups. Although the controls recorded lower SfG than the cadmium exposed mussels, the data shows that the average SfG decreased numerically with increasing concentration of cadmium exposure. Unlike the case of the intertidal mussels which showed declining condition over time, the farm mussels maintained condition over the fourteen days of the exposure. Overall, the rates of clearance, respiration, excretion and SfG were low for the farm mussels compared to the intertidal specimens.

Comparisons between farm and intertidal mussels

Physiological measurements as well as the condition indices were substantially different for farm and intertidal mussels. For the clearance and SfG (Table 5.11), the rates recorded for the farm mussels were considerably lower than the rates for the intertidal mussels. On the other hand, the condition of the farm mussels was higher than for the intertidal mussels (Table 5.12).

These differences in physiological rates as well as condition could have been caused by differences in the body mass of the intertidal and farm mussels which were of the same shell lenght.

Table 5.11: Experimental average SfG (J g^{-1} hr^{-1}) of farm and intertidal mussels exposed to low levels of cadmium.

Treatment	Farm	Intertidal
0 µg Cd L^{-1}	2.76	17.87
33 µg Cd L^{-1}	4.73	14.74
66 µg Cd L^{-1}	4.23	13.83
99 µg Cd L^{-1}	3.58	14.89

Table 5.12: Experimental average condition index of farm and intertidal mussels exposed to low levels of cadmium.

Treatment	Farm	Intertidal
0 μg Cd L^{-1}	144.58	87.31
33 μg Cd L^{-1}	170.20	91.39
66 μg Cd L^{-1}	158.61	97.08
99 μg Cd L^{-1}	168.06	93.67

The mean dry weights of the soft tissue were 0.77 g and 1.89 g for the intertidal and farm mussels respectively. This difference in weight could represent a number of factors including better access to food, longer growth period or better growth efficiencies which resulted from the different growing conditions encountered by the two populations before the experiments were conducted. Consequently, by calculating the physiological rates on a dry weight basis, clearance and SfG were reflecting conditions which the mussels were exposed to for the months prior to the fourteen day experiments reported here. However, even if the differences in tissue dry weight are taken into account, the observed differences in SfG are still difficult to understand. This suggests that there are real differences in the physiology of farm and intertidal *P. canaliculus*.

In addition to the variability caused by differences in dry weight, it is possible that these unexpected results could have been because the farm mussels were more susceptible to the unnatural laboratory conditions. The low range recorded in most of the physiological measurements conducted on the farm mussels suggests that these specimens would not be as good as the intertidal mussels in terms of teasing out the effects of low concentrations of cadmium from the effects of general environmental stress which occur in seawater environments. The low clearance, high excretion and high respiration rates of the controls in the farm mussel experiments suggest this. In the experiment using farm mussels, mussels in the control treatment started spawning soon after the start of the experiment. Interestingly, mussels in the 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1} treatments did

not spawn at this time. These results suggest that in the case of farm mussels, there was overlap between general experimental stress and the stress caused by the chemical exposure. This could have been a considerable source of variability in the current experiments.

Conclusions

The experiments reported here show that the SfG of *P. canaliculus* mussels was affected by seawater containing 500 μg Cd L^{-1}, a concentration which would be classified as acute exposure (USEPA, 2001). Results of the second two experiments show that farm and intertidal *P. canaliculus* were not affected by levels of cadmium of 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1}. Part of the reason for few significant results may be that transfer of the farm mussels from the field to the aquarium to the temperature control rooms may have caused a high level of background stress which masked the stress caused by the cadmium treatments.

The results of the present study are similar to results obtained on *Mytilus edulis* which showed that 11.24 μg Cd L^{-1} did not affect respiration, clearance or scope for growth of *Mytilus edulis* (Vercauteren and Blust, 1999). Also, SfG of the freshwater snail *Brotia hainanensis* was impaired only at concentrations exceeding 800 μg Cd L^{-1}. In that study, respiration was never affected at concentrations ranging from 400 μg Cd L^{-1} to 3,000 μg Cd L^{-1}. In another study, when juvenile *Callinectes sapidus* crabs were exposed to 50 μg Cd L^{-1} and 100 μg Cd L^{-1} at a salinity of 25 ppt, SfG was not significantly affected by either concentrations after twenty-one days of exposure (Guerin and Stickle, 1999). However, other studies have shown that 150 μg Cd L^{-1} affected respiration in *P. viridis* (Cheung and Cheung, 1995). Also, at 160 μg Cd L^{-1}, the SfG of the gastropod *Nassarius festivus* was depressed (Wo *et al.*, 1999).

The methodology used in the present study utilized 100% of exchange of seawater on each day in order to avoid the accumulation of toxic metabolites in the 37 L exposure tanks. This is a unique type of methodology as most previous studies have been conducted using small exposure containers with only partial daily exchange of media. The fact that 100% exchange was carried out daily means that much of the Cd^{+2} applied in the stock solution

may have reacted with chloride on a daily basis, minimizing the amount of free ions in full seawater.

Such complexation reactions are likely to have become less influential at concentrations approaching 1,000 μg Cd L^{-1}. Consequently, although this level of cadmium exposure did not cause mortality at full salinity, clearance rates and SfG were impaired. By comparison, 1,500 μg Cd L^{-1} caused over 80% mortality and depression of the respiration rates of the surviving mussels in *P. viridis* (Cheung and Cheung, 1995).

Results from the present study show that *P. canaliculus* exposed to cadmium at concentrations below 1,000 μg Cd L^{-1} did not show altered clearance, respiration, excretion, SfG or condition. This concentration of cadmium is lower than the minimum concentration of 7,500 μg Cd L^{-1} which has previously been shown to affect *Mytilus edulis* (Redpath and Davenport, 1998). It is also possible that the toxic effects of cadmium on *P. canaliculus* is affected by the salinity of the marine environment where this species is found.

Chapter 6 Effects of reduced salinity and cadmium on farmed and intertidal *Perna canaliculus* mussels

6.1 Introduction

Increases in population and industrial output at numerous locations in the world have caused the background concentration of cadmium in aquatic ecosystems to increase (Nriagu, 1989). This global trend has been quantified in the Mediterranean (UNEP/FAO/WHO, 1989), North America (Page *et al.*, 1987), the Baltic (Broman *et al.*, 1991), Oceania (Bennet-Chambers *et al.*, 1997) and even the Arctic (Dietz *et al.*, 1998). These background levels of cadmium in the natural environment have been attributed to effluents as well as indirect sources such as deposition (OSPAR, 2004) from urban and industrial fuel combustion.

Although elevated background levels of cadmium in the environment have been shown to be a global trend, some of the highest concentrations of cadmium in mussel tissue have been recorded at low salinity environments (Fischer, 1986; Cossa, 1988). For example, *Mytilus edulis* mussels in the southern Bothnian sea (salinity 5.5 ppt) contained 9.7 mg Cd kg^{-1} (dry weight) compared to 3.8 mg Cd kg^{-1} (dry weight) in the Baltic Sea proper (salinity 7 ppt). This gradient was not evident for zinc which ranged from 132 mg Zn kg^{-1} to 117 mg Zn kg^{-1} for the same locations (Broman *et al.*, 1991). One explanation offered was that cadmium was more readily accumulated in low salinity seawater.

In addition to being an important factor in the modulation of the effects of cadmium and other toxins on bivalves, salinity plays important roles which affect some of the basic physiological indices of bivalves. These include effects on clearance, respiration and excretion of bivalves. On model has shown that oxygen consumption increased as salinity decreased from 30 ppt to 10 ppt in *Mytilus edulis* (Stickle and Sabourin, 1979). Another pattern recorded for *M. edulis* was increased respiration in isolated gill tissues at salinities between 28 ppt and 20 ppt compared to 30 ppt (Bayne *et al.*, 1976a). Increased respiration and excretion in *Argopecten purpuratus* kept at 12°C were also seen as salinity decreased from 30 ppt to 24 ppt (Navarro and Gonzalez, 1998). However, as salinities approached 15 ppt or lower, respiration and excretion rates decreased below the rates noted at 30 ppt (Navarro and Gonzalez, 1998). On the other hand, decreased salinity has not been shown to increase clearance in bivalve. For example, clearance rate decreased with salinity and was significantly lower at 24 ppt compared to 30 ppt (Navarro and Gonzalez, 1998). In contrast to the behaviour of the clearance, respiration and excretion rates, absorption efficiency in bivalves was not affected by reduced salinity, even when reduced salinity affected clearance rate (Sobral and Widdows, 1997b; Navarro and Gonzalez, 1998).

The overall effect of reduced salinity on bivalves such as the scallops (eg. *Argopecten purpuratus*) which do not possess the capacity to tightly isolate themselves from the ambient water was that growth rate or scope for growth were reduced (Navarro and Gonzalez, 1998). Conversely, the decline of SfG as a result of low salinity was not as steep in mussels as was the case for scallops (Navarro and Gonzalez, 1998) because mussels were able to seal their valves and protect themselves from the low salinity seawater for extended periods (Davenport, 1981; Navarro, 1988).

These direct effects of salinity on clearance, respiration, excretion and SfG illustrate that there is considerable room in which uncontrolled sources of variability can affect the physiological responses of mussels exposed to trace metals such as cadmium. It is possible that such factors could have contributed to unexpected results in field studies which sought to correlate SfG with cadmium in natural waters (Anderlini, 1992). In that study, concentration of cadmium in mussels was positively related to SfG but copper correlated negatively with SfG.

In laboratory studies, the effects of cadmium on the physiology of various bio-monitors have been variable. For example, numerous studies have suggested no significant effects

of very high concentrations of cadmium on some aquatic invertebrates. These include studies in which 100 µg Cd L^{-1} and 200 µg Cd L^{-1} did not (Forbes and Depledge, 1992) affect feeding or absorption rate in mud snails (*Hydrobia ulvae*) or clearance rate of *Mytilus edulis* mussels (Poulsen *et al.*, 1982). Respiration of *Mytilus edulis* was not affected by 3 µg Cd L^{-1} (Mubiana and Blust, 2007) but *Leptomysis lingvura* suffered metabolic decline at concentrations of 50 µg Cd L^{-1} (Gaudy *et al.*, 1991). No significant effects of 50 µg Cd L^{-1} were evident in *Crassostrea gigas* larvae at 20 ppt (Robert and His, 1985). These examples of apparent tolerance to cadmium by aquatic wildlife may be part of the reason why the ecological (Sonne-Hansen *et al.*, 2002) and human (Satarug *et al.*, 2003) health effects of cadmium are not fully understood (Järup *et al.*, 2000; Knap *et al.*, 2002). In comparison to the comprehensive literature on the effects of toxicants such as tributyl-tin (TbT), the lack of information on the effects of cadmium on wildlife need for a more comprehensive risk assessment of cadmium in the environment (Burger, 2008).

Most studies have focused on accumulation rates and lethal concentrations of cadmium. These studies are generally conducted under static conditions in laboratory settings. These conditions may not provide a complete representation of the effects of toxic trace metals such as cadmium which may occur in natural settings. One variable which may be an important modulator of the effects of cadmium on coastal wildlife is salinity (De Wolf *et al.*, 2004). Higher salinity is believed to protect animals from the harmful effects of cadmium but much research remains to be conducted to demonstrate how indices such as SfG may differ under different conditions. This study was therefore designed to investigate how reduced salinity affected the physiological responses of wild and cultivated *Perna canaliculus* challenged with 1,500 µg Cd L^{-1}.

6.2 Methods

List of experiments

Experiment 1: Farm mussels exposed to 1,500 µg Cd L^{-1} at 17 and 34 ppt.

Experiment 2: Intertidal mussels exposed to 1,500 µg Cd L^{-1} at 17 and 34 ppt.

Experiment 1: Farm mussels exposed to 1,500 µg Cd L^{-1} at 17 and 34 ppt.

Experimental design

Farm mussels from Pigeon Bay were exposed to four combinations of 34 ppt and 17 ppt salinity seawater with two levels of cadmium (0 μg Cd L^{-1} and 1,500 μg Cd L^{-1}) exposure. Responses in clearance, respiration and excretion rates as well as condition index were measured after three, seven and fourteen days of exposure. The statistical methods used to determine the effects of salinity, cadmium and time have been previously described in Section 2.10.

Collection and maintenance

Farm mussels of shell lengths 5.9-7 cm were delivered in styrofoam containers via same courier from Pigeon Bay to SBS on 15 August, 2007. On the day after arrival of the mussels they were brushed clean and glued to plastic plates using cyanoacrylate as described in Section 2.3 The mussels were then suspended in rapidly flowing seawater to remove traces of the cyanoacrylate glue that might have remained. The flow of seawater was then reduced and the mussels were held for five days before the start of the experiment. During this time, mussels were fed once per day using frozen concentrated *Tetraselmis chuii* which was thawed and dosed into the buckets. Mussels were kept in slowly flowing seawater held at 13°C during this time.

Experimental setup

Four exposure systems were assembled to accommodate four treatments combining two salinities and two concentrations of cadmium (Section 2.5). These treatments were (1) 34 ppt/0 μg Cd L^{-1} (2) 17 ppt/ 0 μg Cd L^{-1} (3) 34 ppt/1,500 μg Cd L^{-1} and (4) 17 ppt/ 1,500 μg Cd L^{-1}. The experiment was conducted at 15°C and lasted for 20 days starting on August 21. Twenty four mussels were stocked in each tank to allow a sample of n = 8 mussels to be sampled after three, seven and fourteen days of exposure to the four treatments. All four exposure systems initially contained full salinity seawater but the salinity in two of the systems was decreased by 3.4 ppt on a daily basis for five days. This ensured that a salinity of 17 ppt was achieved in two of the systems after five days. After 17 ppt salinity was achieved in two of the four experimental tanks, twelve mussels from

each tank were removed and transferred to two tanks in the aquarium room for one day. During this day, exposures to cadmium at normal oceanic salinity and low salinity were started. This was done using the gravity flow method previously described in Section 2.5. Exposure to cadmium at the desired concentration was achieved using an aliquot of 1 ml of a stock which contained 11.27 g of $CdCl_2 2.5 H_20$ in 100 ml dH_20. At the start of the next day, the twelve mussels which had been removed from each tank were returned to the tanks from which they had been taken. Mussels were fed as usual and cadmium spiked water exchanged to the exposure tanks. After three, seven and fourteen days of exposure, evaluations of clearance, respiration, excretion and condition was carried out on four mussels per treatment per day for two succeeding days, based on the staggerd stocking described above. At the end of each day, mussels were shucked and placed to dry in a 55°C oven. This allowed calculation of clearance, respiration, excretion and condition on a dry weight basis before statistical analysis.

Experiment 2: Intertidal mussels exposed to 1,500 μg Cd L^{-1} at 17 and 34 ppt.

Experimental design

Naturally occurring *P. canaliculus* mussels were collected from the low intertide at Taylor's Mistake and were exposed to four treatments. These four treatments comprised of combinations of two levels of cadmium (0 μg Cd L^{-1} and 1,500 μg Cd L^{-1}) with two salinities (34 ppt and 17 ppt). The effects of the four treatments were evaluated after three, seven and fourteen days of exposure. This was done on two successive days based on the staggered stocking of n = 4 mussels per treatment on each day. Data were treated as in Section 2.10.

Collection and maintenance

Intertidal mussels for this experiment were collected on the 21st December 2007 from Taylors Mistake. These mussels were selected for a size range of 5.9-7 cm in the field and transported to SBS in buckets without water. Upon arrival at SBS, the mussels were placed in the SBS aquarium which is maintained at 13°C. The mussels were cleaned of

barnacles and epibionts and attached to plastic plates with cyanoacrylate glue the following day. These attached mussels were then placed in rapidly running seawater to remove traces of the cyanoacrylate. After this, the mussels were suspended in slowly running seawater for two days before start of the experimental exposures.

Experimental setup

On the 26 th December, 2007, ninety eight attached mussels were transferred from the SBS aquarium to the four exposure systems (Section 2.5) which had been filled with normal seawater. The mussels were stocked at a rate of twenty four mussels per tank and were fed. The water in each tank was exchanged daily in a way such that two of the systems were maintained with full salinity seawater for the subsequent five days while the salinity in two of the tanks was reduced to 17 ppt over the five days. This was done by successively replacing 3.7 L of seawater with 3.7 L freshwater in the two low salinity treatment tanks. Mussels in the tanks were fed with thawed *Tetraselmis chuii* on a daily basis. When the salinity of two of the tanks reached 17 ppt, twelve mussels were removed from each tank and temporarily placed in two containers containing 34 ppt and 17 ppt salinity seawater. The mussels which remained in the exposure tanks were then fed as before and exposed to the cadmium by applying 1 ml of a stock which contained 11.27 g of $CdCl_2 2.5\ H_20$ dissolved in 100 ml dH2O to the header and the exposure tanks. Gravity flow was used to exchange 37 L of fresh spiked seawater which displaced 37 L of spent seawater over 20 hours in the exposure tanks. On the second day, the twelve mussels which had been removed a day earlier were returned to the four tanks. On the second and subsequent days, the cadmium stock was only added to the header tank as the exposures proceeded.

Evaluations of clearance, respiration, excretion and condition index were done after three, seven and fourteen days of exposure.

6.3 Results

Experiment 1: Farmed mussels exposed to 1,500 µg Cd L^{-1} at 34ppt and 17 ppt.

Clearance rate

The clearance rates for the control mussels were consistently higher than rates for all other treatments which were generally similar (Figure 6.1). Between days three and fourteen, clearance by the 17 ppt/0 µg Cd L^{-1} treatment increased marginally. The 17 ppt / 1,500 µg Cd L^{-1} treatment showed low clearance throughout the experiment.

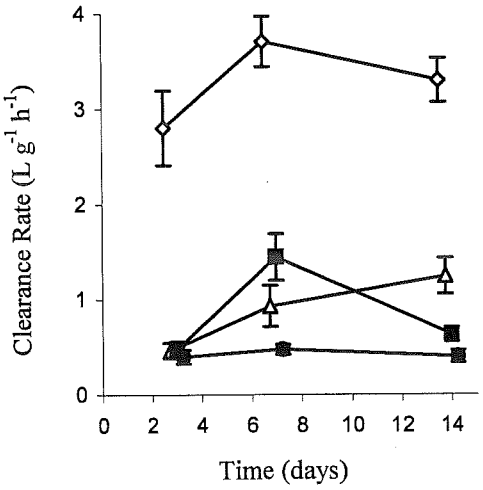


Figure 6.1: Clearance rate +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Repeated measures analysis of variance showed the effects of cadmium, salinity, duration of exposure and the interaction between salinity and cadmium concentration were all significant (Table 6.1).

Table 6.1: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on clearance rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.099	1	0.099	144.3	<0.001
Cadmium Concentration	0.103	1	0.103	150.5	<0.001
Duration of exposure	0.013	2	0.006	10.7	<0.001
Salinity*Cadmium concentration	0.046	1	0.046	67.3	<0.001
Salinity*Duration of exposure	0.005	2	0.003	4.5	0.015
Cadmium Concentration*Duration of exposure	0.003	2	0.001	2.5	NS
Salinity*Cadmium Concentration*Duration of exposure	0.001	2	<0.001	0.7	NS

Post hoc comparisons of the means using Tukey's HSD test confirmed that the difference between the 0 μg Cd $L^{-1}$and 1,500 μg Cd L^{-1} treatments were significant and that the difference between the two salinity treatments was also significant. In terms of significant changes in clearance in a specific treatment over time, the *post hoc* Tukey's HSD test showed a significant increase in clearance between days three and seven.

Analysis of the day three data with ANOVA showed significant differences among the four treatments on days three (ANOVA $F_{(3,28)} = 35.88$, $p < 0$ 0.001), seven (ANOVA $F_{(3,28)} = 44.52$, $p < 0.001$) and fourteen (ANOVA $F_{(3,28)} = 62.84$, $p < 0.001$) because of the high rates recorded for the controls.

Respiration rate

Respiration rates for the 34 ppt/0 μg Cd L^{-1}, 34ppt/1,500 μg Cd L^{-1} and 17 ppt/0 μg Cd L^{-1} treatments were very similar for the duration of the experiment (Figure 6.2). These values were considerably higher than rates recorded for the 17 ppt/1,500 μg Cd L^{-1} treatment which remained low throughout the experiment.

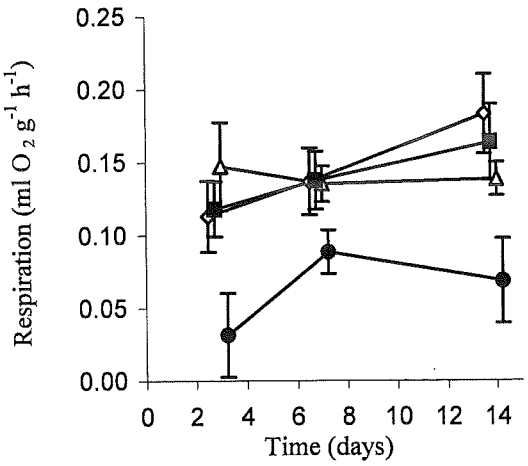


Figure 6.2: Respiration rate +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis of the data trends with repeated measures showed that the effects of salinity and cadmium exposure were significant while the effect of duration of exposure was not significant (Table 6.2).

Table 6.2: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on respiration rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	<0.001	1	<0.001	8.667	0.006
Cadmium Concentration	<0.001	1	<0.001	8.952	0.006
Duration of exposure	<0.001	2	<0.001	2.597	NS
Salinity*Cadmium concentration	<0.001	1	<0.001	7.095	0.013
Salinity*Duration of exposure	<0.001	2	<0.001	1.254	NS
Cadmium Concentration*Duration of exposure	<0.001	2	<0.001	0.532	NS
Salinity*Cadmium Concentration*Duration of exposure	<0.001	2	<0.001	0.843	NS

Post hoc evaluation of results from the repeated measures ANOVA confirmed that reduced salinity and exposure to cadmium caused low respiration rates. Further *post hoc* Tukeys HSD tests showed that the respiration recorded for the 17 ppt/1,500 µg Cd L^{-1} treatment on day three was significantly lower than rates recorded for the 34 ppt/0 Cd L^{-1} treatment on days three, seven and fourteen and the 34 ppt/1,500 µg Cd L^{-1} treatment on day seven. These results indicate that metabolism in farm mussels subject to cadmium exposure at low salinity was depressed from as early as three days of exposure to these conditions.

ANOVA conducted on the data for the separate days showed significant differences among the treatments on days three (ANOVA $F_{(3,28)} = 3.68$, $p = 0.02$) and fourteen (ANOVA $F_{(3,28)} = 4.20$, $p = 0.01$). These were because of the low respiration recorded for the 17 ppt/1,500 µg Cd L^{-1} treatment. Average respiration for this treatment was 0.06 ml O_2 g^{-1} h^{-1} for the duration of the experiment while the other three treatments averaged 0.14 ml O_2 g^{-1} h^{-1}.

Excretion rate

The excretion rates recorded for the four treatments were similar on day three when the average was 35.5 ± 5.65 µg NH_4^+ g^{-1} hr^{-1} (Figure 6.3).

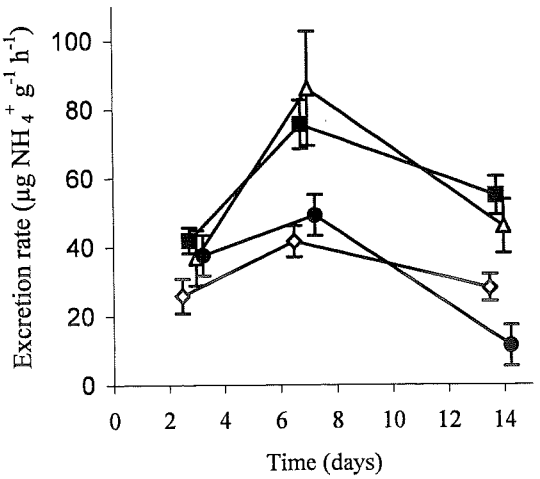


Figure 6.3: Excretion rate +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

After this, two sets treatments with comparatively higher and lower averages developed between days seven and fourteen (Figure 6.3). In the higher group, excretion in the 34 ppt/1,500 μg Cd L^{-1} and the 17 ppt/0 μg Cd L^{-1} treatments increased to 75.5 μg NH_4^+ g^{-1} hr^{-1} and 85.9 μg NH_4^+ g^{-1} hr^{-1} respectively on day seven then both declined slightly on day fourteen. The second trend comprised the controls and the 17 ppt/1,500 μg Cd L^{-1} treatments in which excretion rose only slightly between days three and seven. Similar to the first trend, the increase between days three and seven was followed by a decline on day fourteen. The excretion rate of 31.82 μg NH_4^+ g^{-1} hr^{-1} for the control was the lowest for the experiment.

Evaluation of the data with repeated measures ANOVA showed that neither the effects of neither salinity nor cadmium were significant. However, the effects of duration of the exposures were significant (Table 6.3). The effects of the interaction between salinity and concentration of cadmium were also significant (Table 6.3).

Table 6.3: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on excretion rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	NS	1	NS	NS	NS
Cadmium Concentration	<0.001	1	<0.001	<0.001	NS
Duration of exposure	3.642	2	1.821	30.05	<0.001
Salinity*Cadmium concentration	3.873	1	3.873	22.94	<0.001
Salinity*Duration of exposure	0.785	2	0.393	6.48	0.003
Cadmium Concentration*Duration of exposure	0.529	2	0.264	4.36	0.017
Salinity*Cadmium Concentration*Duration of exposure	0.942	2	0.471	7.77	0.001

Post hoc evaluation of the results of the repeated measures ANOVA with Tukey's test confirmed the significant differences due to duration of exposure were because the average excretion of 63.07 ± 9.45μg NH_4^+ g^{-1} hr^{-1} on day seven was significantly greater than the average of 35.53 ± 5.65 μg NH_4^+ g^{-1} hr^{-1} for day three and 34.98 ± 5.73μg NH_4^+ g^{-1} hr^{-1} for day fourteen.

Analysis of variance showed that on day three, no difference (ANOVA $F_{(3,28)} = 1.86$, $p = 0.16$) was detected among the excretion rates of the four treatments. This changed on day seven (ANOVA $F_{(3,28)} = 4.88$, $p = 0.007$) when excretion by the 17 ppt/0 µg Cd L^{-1} treatments was higher than for the controls. On day fourteen, differences among the treatments were again significant, (ANOVA $F_{(3,28)} = 13.83$, $p < 0.001$) with the 17 ppt/1,500 µg Cd L^{-1} treatments excreting at rates significantly lower than the control and all other treatments.

SfG

Exposure to reduced salinity or cadmium resulted in low SfG compared to the consistent high rates recorded for the controls (Figure 6.4).

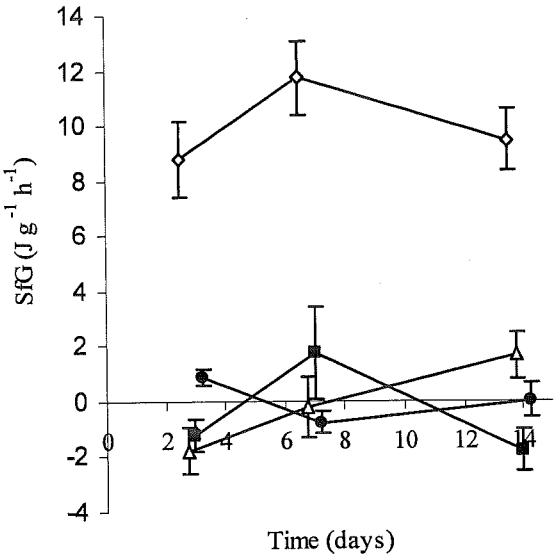


Figure 6.4: SfG +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis of the dataset with repeated measures ANOVA showed that the effects of salinity and cadmium were significant (Table 6.4) while the changes in SfG over time were not significant. *Post hoc* evaluation of the significant effects confirmed that that reduced salinity and exposure to cadmium both caused reduced SfG. In most cases this was because of the high SfG for the controls.

Table 6.4: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on SfG of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.895	1	0.895	85.2	<0.001
Cadmium Concentration	1.016	1	1.016	96.8	<0.001
Duration of exposure	0.056	2	0.028	2	NS
Salinity*Cadmium concentration	1.193	1	1.193	113.6	<0.001
Salinity*Duration of exposure	0.154	2	0.077	5.6	0.006
Cadmium Concentration*Duration of exposure	0.078	2	0.039	2.8	NS
Salinity*Cadmium Concentration*Duration of exposure	0.048	2	0.024	1.7	NS

The effects of the interaction between salinity and cadmium was also found to be significant (Table 6.4). This was because, while SfG of the cadmium exposed and unexposed low salinity treatments were similar, the SfG of the cadmium exposed and unexposed mussels in high salinity were disparately different.

Analysis of variance for SfG on day three showed a significant (ANOVA $F_{(3,28)} = 35.15$, $p < 0.001$) difference due to the high SfG of the controls as well as significant differences between the 17 ppt/1,500 µg Cd L^{-1} treatment and the 17 ppt/0 µg Cd L^{-1} treatments (Tukeys HSD, $p = 0.047$). By day seven, the significant difference (ANOVA $F_{(3,28)} = 27.76$, $p < 0.001$) was only between the controls and one group comprising all the other treatments. However, on day fourteen, the significant difference (ANOVA $F_{(3,28)} = 30.22$, $p < 0.001$) included differences between the 17 ppt/0 µg Cd L^{-1} and the 34 ppt/1,500 µg Cd L^{-1} treatments.

Condition index

The data presented suggested that on the three sampling days, there were no major differences recorded among the controls and the three treatments (Figure 6.5).

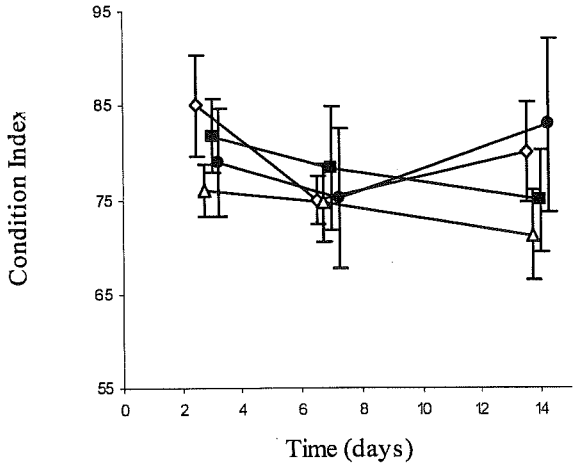


Figure 6.5: Condition index +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis of the data with repeated measures ANOVA did not detect any significant difference due to salinity, cadmium or time of exposure. No significant effects of interactions between salinity and cadmium were detected (Table 6.5). This shows that farm mussels exposed to cadmium did not lose condition due to adverse condition over the fourteen day exposure.

Table 6.5: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on condition index of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.025	1	0.025	0.843	NS
Cadmium Concentration	0.002	1	0.002	0.063	NS
Duration of exposure	0.047	2	0.024	1.085	NS
Salinity*Cadmium concentration	0.02	1	0.02	0.666	NS
Salinity*Duration of exposure	0.009	2	0.005	0.207	NS
Cadmium Concentration*Duration of exposure	0.003	2	0.001	0.068	NS
Salinity*Cadmium Concentration*Duration of exposure	0.035	2	0.018	0.803	NS

These results were consistent with ANOVA which showed no differences among the treatments on days three (ANOVA $F_{(3,28)} = 0.72$, $p = 0.55$), seven (ANOVA $F_{(3,28)} = 0.09$, $p = 0.96$) or fourteen (ANOVA $F_{(3,28)} = 0.54$, $p = 0.66$). Also, there was no indication of any consistent increase or decrease in condition among the three sampling dates.

Experiment 2: Intertidal mussels exposed to 1,500 μg Cd L^{-1} Cadmium at 34 and 17 ppt.

Clearance rate

Clearance rates for the controls were initially high and increased steadily over the duration of the experiment while clearance for the 17 ppt/0 μg Cd L^{-1} treatment only increased between days three and seven. Clearance for the two other treatments remained low for the duration of the experiment (Figure 5.6).

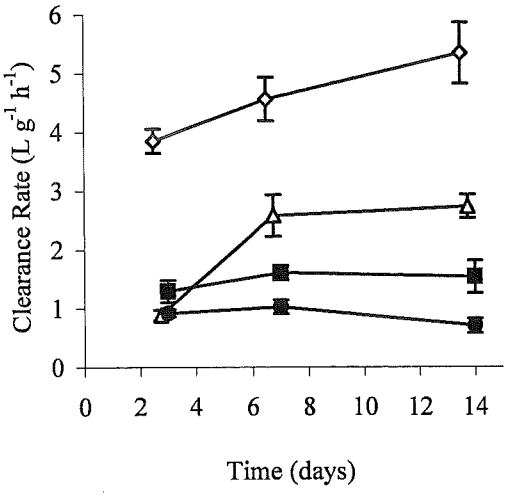


Figure 6.6: Clearance rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis of the data with repeated measures showed that salinity, cadmium level, and duration of the exposures all exerted significant influences on clearance (Table 6.6). The interaction between salinity and concentration of cadmium was also significant.

Table 6.6: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on clearance rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.089	1	0.089	78.4	<0.001
Cadmium Concentration	0.17	1	0.17	150.2	<0.001
Duration of exposure	0.02	2	0.01	10.1	<0.001
Salinity*Cadmium concentration	0.031	1	0.031	27	<0.001
Salinity*Duration of exposure	0.001	2	0.001	0.7	NS
Cadmium Concentration*Duration of exposure	0.017	2	0.008	8.5	0.001
Salinity*Cadmium Concentration*Duration of exposure	0.003	2	0.002	1.6	NS

The *post hoc* Tukey's test confirmed that low salinity and exposure to cadmium reduced clearance in these intertidal mussels. The *post hoc* test also showed that the effect of

duration of exposure was because clearance increased on days seven compared to day three. The interaction detected was because the exposures conducted at the 34 ppt salinity cleared at 3.03 $\pm$ 0.28 L g^{-1} h^{-1} while average clearance for the 17 ppt treatments was 1.47 $\pm$ 0.16 L g^{-1} h^{-1} Similarly, the cadmium exposed mussels cleared at 1.18 $\pm$ 0.15 L g^{-1} h^{-1} while the cadmium naive mussels cleared at 3.32 $\pm$ 0.29 L g^{-1} h^{-1}.

These results concur with ANOVA which showed that on day three, the highest clearance was recorded for the controls while the other treatments cleared at significantly (ANOVA $F_{(3,28)} = 89.87$, $p < 0.001$) lower rates. The rates recorded for the four treatments were also significantly different on days seven (ANOVA $F_{(3,28)} = 35.57$, $p < 0.001$) and fourteen (ANOVA $F_{(3,24)} = 32.50$, $p < 0.001$). On both days, clearance by the controls was different from all other treatments. Also, the 17 ppt/0 µg Cd L^{-1} treatment cleared at rates greater than the 17 ppt/1,500 µg Cd L^{-1} treatment on both days seven and fourteen.

Respiration rate

The rates on day three were initially high but declined sharply on day seven at which time the rates were quite similar. However, the averages increased for three treatments on day fourteen and variability among the treatments also increased at this time (Figure 6.7).

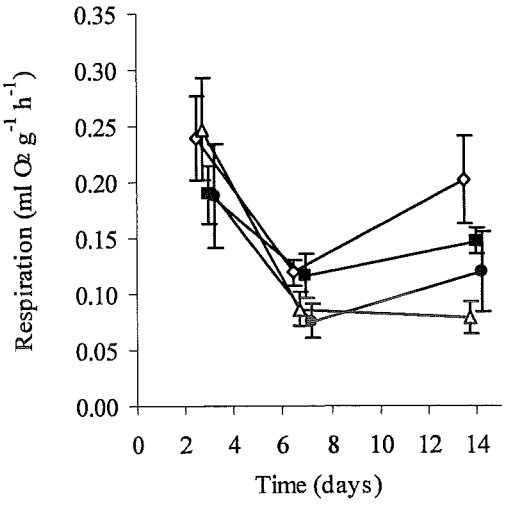


Figure 6.7: Respiration rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

As a result of the consistent similarity among the averages, the repeated measures ANOVA showed that there were no effects of either salinity or cadmium on respiration rates (Table 6.7).

Table 6.7: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on respiration rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	<0.001	1	<0.001	2.53	NS
Cadmium Concentration	<0.001	1	<0.001	0.75	NS
Duration of exposure	<0.001	2	<0.001	17.84	<0.001
Salinity*Cadmium concentration	<0.001	1	<0.001	1.07	NS
Salinity*Duration of exposure	<0.001	2	<0.001	2.41	NS
Cadmium Concentration*Duration of exposure	<0.001	2	<0.001	0.9	NS
Salinity*Cadmium Concentration*Duration of exposure	<0.001	2	<0.001	0.75	NS

However, the decline in respiration between days three and seven was shown to be significant (Table 6.7). *Post hoc* evaluation of the repeated measures ANOVA using Tukey's test showed that the day seven average respiration rate of 0.10 ± 0.02 ml O_2 g^{-1} h^{-1} was significantly lower than the 0.22 ± 0.04 ml O_2 g^{-1} h^{-1} recorded on day three but was similar to the 0.14 ± 0.02ml O_2 g^{-1} h^{-1} recorded on day fourteen.

Analysis of the data on separate days also showed no significant differences among the treatments on days three (ANOVA $F_{(3,28)} = 0.65$, $p = 0.59$) or seven (ANOVA $F_{(3,28)} = 1.85$, $p = 0.16$). Variation among the treatments increased by day fourteen and significant differences were detected (ANOVA $F_{(3,24)} = 4.25$, $p = 0.015$) at that time. Specifically, respiration in the 17 ppt/0 µg Cd L^{-1} treatment was low compared to the controls.

Excretion rate

Excretion rates determined for the all treatments except the 17 ppt/0 µg Cd L^{-1} treatment were stable for the duration of this experiment (Figure 6.8).

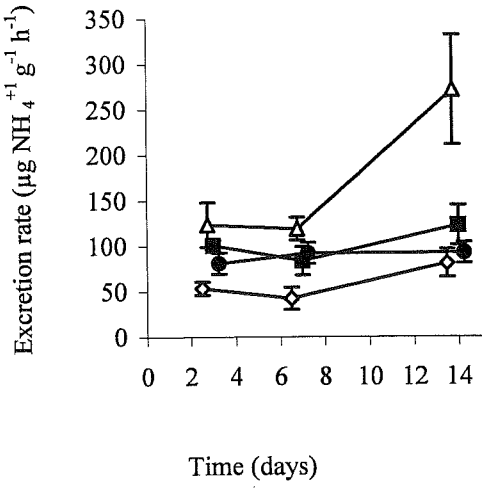


Figure 6.8: Excretion rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Excretion for the 17 ppt/0 µg Cd L^{-1} treatment was high but stable on days three and seven. However, on day 14 excretion in this treatment increased to 271.27 µg NH_4^+ g^{-1} hr^{-1}. The lowest excretion for the experiment was recorded for the controls. The rates recorded for the 17 ppt/0 µg Cd L^{-1} treatment were the highest among the treatments for the duration of the experiment.

Analysis of the trends using repeated measures ANOVA showed that the effects of salinity and duration of exposure were significant (Table 6.8) while the effects of cadmium were not. The interaction of salinity and cadmium concentration was also significant (Table 6.8).

Table 6.8: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on excretion rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	3.057	1	3.057	20.83	<0.001
Cadmium Concentration	0.009	1	0.009	0.06	NS
Duration of exposure	2.355	2	1.177	4.84	0.012
Salinity*Cadmium concentration	3.997	1	3.997	27.23	<0.001
Salinity*Duration of exposure	0.136	2	0.068	0.28	NS
Cadmium Concentration*Duration of exposure	0.651	2	0.326	1.34	NS
Salinity*Cadmium Concentration*Duration of exposure	0.29	2	0.145	0.6	NS

The significant differences detected by the ANOVA were investigated with Tukeys HSD test which confirmed that the mean excretion of 80.35 $\mu g\ NH_4^+\ g^{-1}\ hr^{-1}$ for the mussels in normal salinity was significantly lower than 129.6 $\mu g\ NH_4^+\ g^{-1}\ hr^{-1}$ recorded for the mussels exposed to low salinity. The effect of time was because the average excretion of 114.6 $\mu g\ NH_4^+\ g^{-1}\ hr^{-1}$ for the four treatments on day fourteen was greater than the average rates of 89.2 $\mu g\ NH_4^+\ g^{-1}\ hr^{-1}$ and 83.9 $\mu g\ NH_4^+\ g^{-1}\ hr^{-1}$ on days three and seven respectively.

Analysis of variance of the data for separate days showed that significant differences based on the treatments were found on days three ($F_{(3,28)} = 4.44$, $p = 0.01$), seven ($F_{(3,28)} = 5.22$, $p = 0.005$) and fourteen ($F_{(3,24)} = 6.38$, $p = 0.003$). These differences resulted because excretion in the controls was significantly lower than for the other treatments on each day.

SfG

The SfG values for the controls increased between days three and seven then remained steady between days seven and fourteen. These values were considerably higher than for the other three treatments over the fourteen days of the experiment (Figure 6.9). The 17

ppt/0 µg Cd L^{-1} treatment also showed the trend of increasing SfG but the two cadmium exposed treatments showed low positive or negative SfG values throughout.

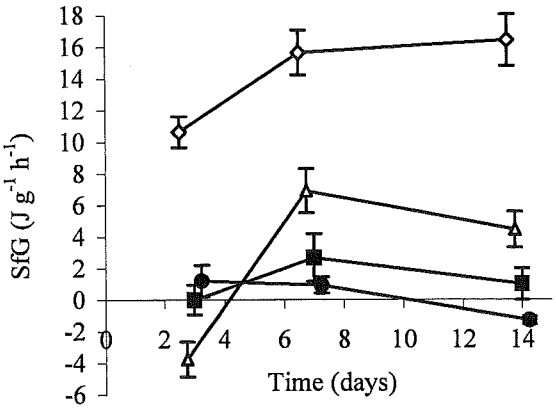


Figure 6.9: SfG +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis of the data with repeated measures ANOVA showed that the effects of salinity, cadmium and duration of exposure were all significant (Table 6.9).

Table 6.9: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on SfG of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	1.27	1	1.27	64.67	<0.001
Cadmium Concentration	1.613	1	1.613	82.14	<0.001
Duration of exposure	0.607	2	0.303	20.55	<0.001
Salinity*Cadmium concentration	0.67	1	0.67	34.11	<0.001
Salinity*Duration of exposure	0.068	2	0.034	2.31	NS
Cadmium Concentration*Duration of exposure	0.311	2	0.156	10.54	<0.001
Salinity*Cadmium Concentration*Duration of exposure	0.169	2	0.085	5.73	0.006

Post hoc analyses of the significant effects with Tukey's test showed that the average of 7.71 J g^{-1} hr^{-1} determined for the full salinity treatments were significantly different from the 1.39 J g^{-1} hr^{-1} calculated for the 17 ppt treatments. The SfG of 8.37 J g^{-1} hr^{-1} for unexposed mussels was different from SfG 0.74 J g^{-1} hr^{-1} for cadmium exposed mussels. The significant effect of duration of exposure was because the difference between the average SfG of 2.01 J g^{-1} hr^{-1} on day three and 6.52 J g^{-1} hr^{-1} on day seven was significant.

In addition to the main effects, the interaction between salinity and cadmium was also significant (Table 6.9). This was because SfG of mussels in the two 17 ppt treatments were of 2.52 $\pm$ 1.21 J g^{-1} hr^{-1} and 0.27 $\pm$ 0.59 J g^{-1} hr^{-1} while mussels in 34 ppt salinity not exposed to cadmium maintained SfG of 14.21 $\pm$ 1.34 J g^{-1} hr^{-1} versus the SfG of 1.21 $\pm$ 1.15 J g^{-1} hr^{-1} for cadmium exposed mussels. This showed that the effects of cadmium were most evident at normal salinity.

Evaluation of the SfG values on day three showed that the differences among the treatments were significant (ANOVA $F_{(3,28)} = 26.88$, $p < 0.001$). The SfG for the 17 ppt/0 µg Cd L^{-1} treatment was significantly lower than the 17 ppt/1,500 µg Cd L^{-1} treatment. On day seven, significant differences were again detected (ANOVA $F_{(3,28)} = 42.58$, $p < 0.001$) but at this time, SfG for the 17 ppt/0 µg Cd L^{-1} treatment was greater than for the 34 ppt/1,500 µg Cd L^{-1} treatment. On day fourteen, significant differences were again detected (ANOVA $F_{(3,24)} = 39.23$, $p < 0.001$). At this time, a significant difference was detected between the 17 ppt/0 µg Cd L^{-1} and the 34 ppt/1,500 µg Cd L^{-1} treatment.

Condition index

Condition indices measured on the four treatments showed variability among the treatments from day three but there were few indication of treatment effects. Overall, the controls showed the highest average condition for days three and seven but these underwent a decline between days seven and fourteen (Figure 6.10).

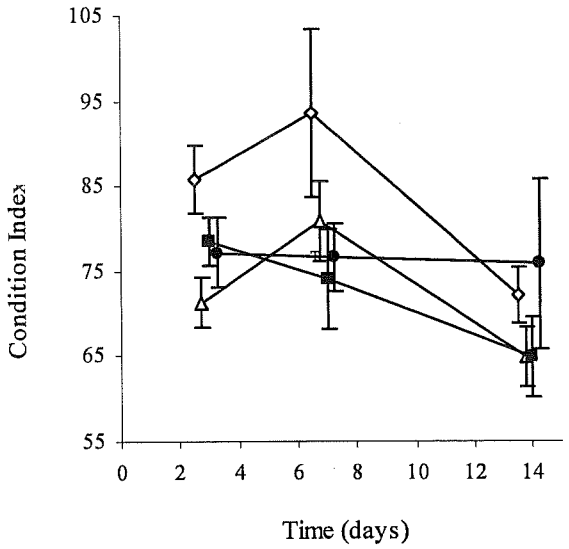


Figure 6.10: Condition index +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cd L^{-1} (◇), 34ppt/1,500 µg Cd L^{-1}(■), 17ppt/0µg Cd L^{-1}(△) and 17ppt/1,500 µg Cd L^{-1} (●) after three, seven and fourteen days.

Analysis with repeated measures ANOVA showed that neither salinity nor cadmium affected condition (Table 6.10). A significant effect of duration of exposure was however detected. An interaction between salinity and cadmium concentration was also detected. The interaction arose because the two treatments which were not exposed to copper showed similar trends in condition over time while the other two treatments reacted differently.

Table 6.10: Repeated measures ANOVA of effects of cadmium, salinity and duration of exposure on condition index of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.009	1	0.009	0.62	NS
Cadmium Concentration	0.009	1	0.009	0.63	NS
Duration of exposure	0.24	2	0.12	5.4	0.008
Salinity*Cadmium concentration	0.151	1	0.151	10.02	0.004
Salinity*Duration of exposure	0.018	2	0.009	0.41	NS
Cadmium Concentration*Duration of exposure	0.049	2	0.024	1.1	NS
Salinity*Cadmium Concentration*Duration of exposure	0.001	2	0.001	0.03	NS

Post hoc analysis with Tukeys HSD test confirmed a significant decline in average condition between days seven and fourteen.

In addition to the repeated measures ANOVA, the lack of significant differences due to salinity or cadmium exposure was reflected by analysis of variance which showed no significant differences among the treatments on day three (ANOVA $F_{(3,28)} = 2.77$, $p = 0.06$), seven (ANOVA $F_{(3,28)} = 1.62$, $p = 0.21$) or fourteen (ANOVA $F_{(3,28)} = 1.05$, $p = 0.39$).

6.4 Discussion

In the natural environment, mussels are subject to constant changes in natural stressors such as temperature, food availability, wave intensity, salinity and dissolved oxygen (Resgalla *et al.*, 2007a). Mussels can also be stressed by pulses of land based contaminants created by human activities (Wang *et al.*, 2005). The SfG index quantifies the intensity of these stresses on an ecosystem by using bivalves as bioindicators. Scope for Growth can be used to differentiate between natural and anthropogenic stressors (Burt *et al.*, 2007). The experiments contained in this chapter investigated how the components of SfG reacted to both normal and anthropogenic type stresses applied simultaneously. Results from experiments which quantified the physiological responses in farm and intertidal *Perna canaliculus* confirmed that cadmium affected the clearance, respiration and SfG of farm mussels as well as clearance, excretion and SfG of intertidal mussels. On the other hand, salinity affected clearance, respiration and SfG of farm mussels and the clearance, excretion and SfG of the intertidal mussels.

Clearance rate

At the start of the experiments, clearance for the intertidal mussels was 3.85 ± 0.20 L g^{-1} h^{-1} and 2.80 ± 0.39 L g^{-1} h^{-1} farm mussel controls. Over the three time intervals, the averages were 4.58 ± 0.37 L g^{-1} h^{-1} for the intertidal mussel controls and 3.27 ± 0.30 L g^{-1} h^{-1} for the farm mussel controls (Table 6.11). This difference in clearance may have been seen in previous research which showed that filtration was slightly greater for high shore *Choromytilus meridionalis* compared to sublittorial specimens (Griffiths, 1980b). The

results of the present study may also be supported by another comparative study which concluded that intertidal mussels displayed higher clearance rates than subtidal specimens when only low levels of ration were available (Riisgård and Randløv, 1981).

In the present study, clearance for the field collected intertidal mussels controls increased over the two weeks of the experiment (Figure 6.6). During these two weeks, the farm mussel controls never showed an increase in clearance (Figure 6.1). A similar result was seen over fifteen days when intertidal and raft cultured *Mytilus galloprovincialis* were transferred to laboratory conditions. The intertidal mussels responded with a greater increase in clearance compared to raft cultured mussels (Labarta *et al.*, 1997).

Table 6.11: Mean clearance rates (L g^{-1} L^{-1}) for farm and intertidal mussels exposed to combinations of 34 ppt and 17 salinity seawater and 0 µg Cd L^{-1} and 1,500 µg Cd L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 µg Cd L^{-1}	3.27 ± 0.30	4.58 ± 0.37
17 ppt/ 0 µg Cd L^{-1}	0.88 ± 0.16	2.06 ± 0.21
34 ppt/ 1,500 µg Cd L^{-1}	0.85 ±0.13	1.48 ± 0.19
17 ppt/ 1,500 µg Cd L^{-1}	0.42 ± 0.06	0.88 ± 0.10

In the present study, the lower clearance of the farm mussels could have been related to the fact that the farm mussels were transferred from submerged ropes to laboratory conditions which could be considered intertidal. In the laboratory, the position of the mussels could not be replicated because the small size of the experimental tanks only permitted mussels to be kept at 25 cm below the surface of the water. In addition to the actual depth of immersion, the feeding regime could have favored intertidal mussels because feeding was done only twice per day, using an algal concentrate. A more appropriate feeding regime could have involved using continuous feeding.

In the present study, clearance by the intertidal mussels subject to the 17 ppt/0 µg Cd L^{-1} treatment also increased significantly from 0.88 L g^{-1} h^{-1} to 2.73 L g^{-1} h^{-1} between days

three and seven. This was not the same for the farm mussels as the slight increase from 0.46 L g^{-1} h^{-1} to 1.24 L g^{-1} h^{-1} by farm mussels at 17 ppt was not significant. The increase in clearance for the controls as well as the mussels in the 17 ppt/0 μg Cd L^{-1} in the intertidal experiment suggests that intertidal mussels had greater capacity to adapt to a salinity of 17 ppt. The current results for clearance displayed by the farm and intertidal mussels may be similar to results of clearance demonstrated in two populations of *Perna viridis* (Blackmore and Wang, 2003). One population was exposed to more constant salinity of 28 ppt – 32 ppt in its habitat while the other population was periodically subjected to low salinities during the summer months. When the clearance of these mussels were evaluated at salinities ranging from 6 ppt to 30 ppt under laboratory conditions, the highest clearance rates were recorded for the populations which had previously been exposed to reduced salinity (Blackmore and Wang, 2003). Adaptation to periodic low salinity allowed these mussels to maintain high clearance rates at low salinities which they were challenged with under laboratory settings. Mussels from sites which had never experienced these low salinities showed low clearance when challenged with low and intermediate low salinities (Blackmore and Wang, 2003).

The current experiments also showed that after three days of exposure, intertidal as well as farm mussels which were subject to 1,500 μg Cd L^{-1} exposure in full salinity seawater recorded low clearance rates. Between days three and seven, the clearance by the farm mussels in this treatment increased temporarily but this declined between days seven and fourteen. There was no parallel temporary increase in the clearance rate of the intertidal mussels exposed to 1,500 μg Cd L^{-1}. This suggests that farm musses were able to cope with cadmium better than farm mussels.

These data show that 1,500 μg Cd L^{-1} was toxic to both farm and intertidal mussels. In previous work, 100 μg Cd L^{-1} had no effect the clearance rate of *Mytilus edulis* (Poulsen *et al.*, 1982) but concentrations between 7,500 – 10,000 μg Cd L^{-1} caused significant decreases in the clearance rate of *Mytilus edulis* (Redpath and Davenport, 1998). The results of the present work suggests that concentrations of 1,500 μg Cd L^{-1} overcame the protective effects which higher salinity confers on marine life. This was the case for both the farm and the wild mussels. Also, clearance in the 17 ppt/ 1,500 μg Cd L^{-1} treatments was always low showing that the physiology of these mussels was severely compromised .

Respiration rate

The respiration rate in farm mussels decreased in response to both reduced salinity and cadmium exposure (Table 6.12). Both effects were due to the low levels of respiration recorded in the 17 ppt/ 1,500 μg Cd L^{-1} treatment. Neither exposure to cadmium or reduced salinity affected respiration rate in intertidal mussels.

Table 6.12: Mean respiration rates (ml O_2 g^{-1} L^{-1}) for farm and intertidal mussels exposed to combinations of 34 ppt and 17 salinity seawater and 0 μg Cd L^{-1} and 1,500 μg Cd L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 μg Cd L^{-1}	0.14 ± 0.02	0.19 ± 0.03
17 ppt/ 0 μg Cd L^{-1}	0.14 ± 0.02	0.14 ± 0.02
34 ppt/ 1,500 μg Cd L^{-1}	0.14 ± 0.02	0.15 ± 0.02
17 ppt/ 1,500 μg Cd L^{-1}	0.06 ± 0.02	0.13 ± 0.03

This was in spite of the fact that respiration in intertidal mussels declined significantly between days three and seven in the intertidal mussels. These findings indicate that respiration rate of farm mussels was more sensitive to cadmium and salinity.

The current results are similar to findings on the response of respiration in the gastropod *Nassarius festivus* exposed to cadmium. At concentrations of 160 μg Cd L^{-1}, 550 μg Cd L^{-1} and 950 μg Cd L^{-1}, respiration was not affected. However, respiration was significantly affected at 1,200 μg Cd L^{-1} (Wo *et al.*, 1999). In the case of the freshwater snail, *Broria hainanensis* respiration was not affected by concentrations between 250 μg Cd L^{-1} and 3,250 μg Cd L^{-1} (Lam, 1996). These were unlike results for the mussel *Perna viridis* exposed to cadmium at concentrations between 320 μg Cd L^{-1} to 1,500 μg Cd L^{-1} (Cheung and Cheung, 1995). Respiration rate of all the treatments including the controls declined between days one and six of the exposure. This decline was similar to the results obtained for the intertidal mussels in the present study. As a result, the only factor which resulted in

significant differences in the respiration rate of intertidal mussels was time (Table 6.7). A similar decline in respiration over time was also noted for intertidal *Mytilus galloprovincialis* held for fifteen days under laboratory conditions (Labarta *et al.*, 1997).

Excretion rate

Results from the two experiments show that excretion rates were higher for the intertidal mussels compared to the farm mussels (Table 6.13). Salinity and cadmium exposure did not significantly affect excretion in farm mussels but low salinity resulted in increased excretion in intertidal mussels. Elevated excretion rates in bivalves is thought to be an indication of catabolism of protein body reserves for maintenance (El-Shenawy, 2004). The results obtained for the intertidal mussels suggests that the protein body mass could have been utilized by mussels in the low salinity treatments. However, this was not reflected in the condition indices of the mussels although significant differences were detected among the dry weights for the four sets of intertidal mussels on day three of the exposure.

Table 6.13: Mean excretion rates (μg NH_4^+ g^{-1} L^{-1}) for farm and intertidal mussels exposed to combinations of 34 ppt and 17 salinity seawater and 0 μg Cd L^{-1} and 1,500 μg Cd L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 μg Cd L^{-1}	31.82 ± 4.46	58.69 ± 11.60
17 ppt/ 0 μg Cd L^{-1}	56.22 ± 10.77	170.96 ± 32.47
34 ppt/ 1,500 μg Cd L^{-1}	57.40 ± 5.432	102.1 ± 14.53
17 ppt/ 1,500 μg Cd L^{-1}	32.66 ± 7.10	88.19 ± 11.62

At that sampling, the dry weights of mussels in low salinity were significantly lower than the dry weights in the high salinity treatments. However, owing to a decline in the dry weights of the mussels in full salinity, the dry weights of all treatments were similar by day

fourteen. This suggests that for the intertidal mussels, body mass was being used to osmoregulate or maintain turgor pressure in the low salinity treatments (Navarro, 1988). If this was indeed the case, it was seen for the intertidal mussels in the 17 ppt/0 μg Cd L^{-1} treatment which excreted high rates of ammonia for the duration of the experiments. This high excretion rate was not linked to a high clearance rate but may have been linked to the low respiration rate of these mussels on day fourteen.

SfG

In the present study, intertidal mussels recorded higher SfG than the farm mussels (Table 6.14). For both farm and intertidal mussels, reduced salinity and exposure to cadmium resulted in significantly lowered SfG. The interactions of salinity with cadmium concentration were also significant for both farm and intertidal mussels.

Table 6.14: Mean SfG for farm and intertidal mussels exposed to combinations of 34 ppt and 17 salinity seawater and 0 μg Cd L^{-1} and 1,500 μg Cd L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 μg Cd L^{-1}	10.00 ± 1.28	14.21 ± 1.34
17 ppt/ 0 μg Cd L^{-1}	-0.14 ± 0.92	2.52 ± 1.21
34 ppt/ 1,500 μg Cd L^{-1}	-0.44 ± 1.01	1.21 ± 1.15
17 ppt/ 1,500 μg Cd L^{-1}	0.03 ± 0.45	0.27 ± 0.59

These were unexpected results because subtidal mussels are known to be faster growing mussels compared to intertidal mussels (Menge *et al.*, 2007; Petes, 2007; Petes *et al.*, 2007). However, if the farm mussels are considered to be subtidal mussels, the results from the current experiment can be considered similar to previous findings (Weatherhead, 1993) for *P. canaliculus*. These results may be based on stress responses demonstrated by mussels transferred from subtidal locations to conditions of intermittent feeding, artificial

lighting, noise, movement, etc. For example, it was noted that farm mussels would close their valves rapidly upon being approached by the experimenter. On the other hand, valve closure by intertidal mussels was generally slow. Previous research has shown that when subtidal *Mytilus edulis* were reared in laboratory mesocosms with only intermittent feeding, the SfG attained by these specimens was lower than for intertidal mussels (Shick *et al.*, 1988). However, when the subtidal specimens were continuously fed, their SfG was higher than SfG of the intertidal specimen (Riisgård and Randløv, 1981; Shick *et al.*, 1988). The higher SfG (Weatherhead, 1993) but slower growth (Petes *et al.*, 2007) of intertidal mussels compared to subtidal specimens has also been attributed to adaptive feeding response to decreased feeding time and starvation which the intertidal specimens have to overcome (Griffiths, 1981). To correct for the difference in feeding times of different specimens, a number of studies have advocated use of a correction factor known as the feeding time activity (FTA). Feeding time activity is the percentage of a 24 hour period spent feeding (Bougrier *et al.*, 1998; Haure *et al.*, 2003).

In the present study, it was evident that both farm and intertidal mussels exposed to 17 ppt were under extreme stress. In the case of the intertidal mussels exposed to low salinity, SfG decreased by 82% while for farm mussels, SfG decreased by over 100%, indicating that the effects of low salinity were much more severe for farm mussels.

Although SfG was severely depressed for both sets of mussels in low salinity, an increase from –3.77 J g^{-1} hr^{-1} to 6.89 J g^{-1} hr^{-1} between days three and seven for intertidal mussels suggests an ability to adapt to low salinity in the short term. This increase was only from –1.81 J g^{-1} hr^{-1} to -0.25 J g^{-1} hr^{-1} for the farm mussels. The increase in SfG of intertidal mussels could reflected a behavioral strategy which intertidal mussels are capable of when faced with challenging conditions. Such behavioral adaptations include gaping (Moon and Pritchard, 1970) at low tide, increasing clearance rates when feed quantity is low (Griffiths, 1980b) or closing valves and suspending feeding when feed concentrations remained low for extended periods (Riisgård and Randløv, 1981). However, the increase in SfG was accompanied by increased excretion in intertidal mussels. This suggested that increased SfG could be considered a physiological acclimation in intertidal mussels.

Interactions

Various factors have been shown to contribute to multiple regression equations describing the the effect of environmental variables on SfG. These factors include body mass, ration, seasonal cycle, ambient temperature (Widdows, 1978a). In addition to these, salinity and duration of exposure have been shown to affect physiological indices measured on bivalves (Munari and Mistri, 2007). In the current experiments, significant effects of cadmium, salinity and time of exposure a number of important interactions between salinity and cadmium were noted. These interactions were significant in three of the five physiological indices measured on the farm and the field mussels.

The effects of the interaction on clearance rate were similar for farm and intertidal mussels. In both cases, the interaction comprised a steeper decline in clearance due to effects of cadmium in high salinity compared to low salinity. However, the F value was much greater for the farm mussels, indicating that these mussels were more prone to the combined effects of cadmium and salinity. Reduced salinity alone has been shown to decrease the SfG of *Thais lapillus* at 25 ppt (Stickle and Bayne, 1987), *Argopecten purpuratus* at 27 ppt (Navarro and Gonzalez, 1998) and *Choromytilus chorus* at 18 ppt (Navarro, 1988). In addition, exposure to cadmium has also been shown to decease SfG of clams at concentrations as low as 100 μg Cd L^{-1} (Neuberger-Cywiak *et al.*, 2007) but SfG of *Mytilus edulis* was not affected at a concentration of 11.24 μg Cd L^{-1} (Vercauteren and Blust, 1999). Clearance, respiration, assimilation efficiency, net growth efficiency or gross growth efficiency were not affected by 10 μg Cd L^{-1} or 100 μg Cd L^{-1} (Poulsen *et al.*, 1982). Similarly, SfG of *Brotia hainanensis* was not affected at 400 μg Cd L^{-1} (Lam, 1996). This tolerance to elevated cadmium by marine mussels in aquatic systems may relate to decreased concentration of the dissolved free cadmium because of complexation by chlorides or carbonates (USEPA, 2003) present in coastal waters. This tolerance to cadmium at high salinity was only seen for the clearance rate of intertidal mussels (Figure 6.6).

In terms of overall physiology of the mussels, reduced salinity caused major decreases in SfG. This may be because *P. canaliculus* is a marine species (Wood *et al.*, 2007) preferring marine intertidal habitats (Menge *et al.*, 2007). Most green lipped mussel farms are located in protected bays along the coastline (Longdill *et al.*) where salinity is constant (Hawkins *et al.*, 1999). However, *P. canaliculus* may also occur in the Hokianga, Kaipara

and Whangarei estuaries (Morrison, 2005) but those occurring in the Whangapoua estuary are said to be small (Moore *et al.*, 2004b). The low SfG obtained for mussels in the 17 ppt treatments in this chapter could be a reflection of this.

These results show that salinity is a critical modulator of how cadmium affects mussel physiology. If the salinity is low, cadmium will only cause slight declines. At full salinity, presence of cadmium will cause dramatic decline in SfG. This is important because, even among different locations in the same estuary such as the Avonhead-Heathcote, salinity can vary between 8 ppt and 30 ppt (Marsden, 2004). It is apparent from these results that exposure of either farm or intertidal *P. canaliculus* to low salinities would severely affect SfG and that acute exposure to cadmium would cause secondary effects. On the other hand, SfG of mussels in normal salinity containing cadmium would be severely affected.

Chapter 7 Effects of reduced salinity and copper on scope for growth of farmed and intertidal *Perna canaliculus*

7.1 Introduction

A number of laboratory studies have shown that copper affects important physiological functions of marine mussels and becomes lethal to species such as *Mytilus galloprovincialis* at concentrations ranging between 12 μg Cu L^{-1} and 53 μg Cu L^{-1} (Moore *et al.*, 1984; Widdows and Johnson, 1988; USEPA, 2003; Arnold *et al.*, 2006). Low concentrations of dissolved copper can also lead to sub-lethal stress in clearance in bivalves (Davenport and Manley, 1978). Because various physiochemical characteristics of seawater may cause similar effects to copper, there is a need for experiments to distinguish between the effects of the metal and environmental factors in order to determine environmental risk (Harding, 2005). Also, environmental factors can be important modulators of copper toxicity and physiological responses in mussels (Widdows *et al.*, 1984). Environmental factors can also affect the uptake rates of metals such as copper and cadmium (USEPA, 2003; Abbe *et al.*, 2003).

Environmental factors which have been shown to influence the physiological effects of copper on bivalves include salinity (Davenport, 1977), temperature (Parry and Pipe, 2004) and dissolved organic matter (USEPA, 2003). For example, physiological indices such as clearance rates in bivalves can be caused by low salinity (Navarro, 1988), low algal food concentrations (Bayne *et al.*, 1993; Riisgård *et al.*, 2003) or elevated seston levels (Hawkins *et al.*, 1999).

In addition to these known interactions between natural and artificial stressors, previous studies have also shown that when molluscs such as *Trochus maculates or Mytilus edulis* were exposed to copper at differing salinities, salinity accounted for much of the differences in biomarker and physiological responses (Elfwing. and Tedengren, 2002; Prevodnik *et al.*, 2007). Reduced salinity may be important for *P. canaliculus* which occupies estuarine (Gardner and Kathiravetpillai, 1997) as well as intertidal and subtidal marine habitats (Sin *et al.*, 1990). The experiments in this chapter were therefore designed to determine how reduced salinity affected the physiological responses of farm and intertidal *P. canaliculus* mussels exposed to low concentrations of copper.

7.2 Methods

List of experiments

Experiment 1: Intertidal mussels exposed to 100 μg Cu L^{-1} at 17 and 34 ppt.

Experiment 2: Farm mussels exposed to 100 μg Cu L^{-1} at 17 and 34 ppt.

Experiment 1: Intertidal mussels exposed to 100 μg Cu L^{-1} at 17 and 34 ppt.

Experimental design

Intertidal *P. canaliculus* mussels were exposed to four treatments combining salinity and copper for durations of three, seven and fourteen days. These treatments were:

1) 34 ppt & 0 μg Cu L^{-1}.
2) 17 ppt and 0 μg Cu L^{-1}.
3) 34 ppt & 100 μg Cu L^{-1}..
4) 17 ppt & 100 μg Cu L^{-1}.

The experiment was stocked with 24 mussels and samples of n=8 mussels were removed after three, seven and fourteen days of exposure. These mussels were evaluated for

clearance, respiration, excretion and condition on a dry weight basis as in Sections 2.6-2.9. The physiological parameters were then analysed using repeated measures ANOVA (as in Section 2.10) to determine the effects of salinity, cadmium and duration of the exposure on the scope for growth.

Collection and maintenance

Two hundred mussels were collected from low intertidal mussel beds north of the bay at Taylor's Mistake on 28 th September, 2007. Only specimens of shell length between 5.9 to 7 cm were selected. These were transferred to the SBS aquarium and left in running water overnight. The following day, mussels were cleaned of algae and other encrustations including barnacles and marine worms. They were individually attached to plastic plates using cyanoacrylate glue, suspended in slowly running seawater for three days a fed once per day with algae concentrate. Mussels were kept in the aquarium for a total of six days before the start of the experiment.

Experimental setup and evaluation

At the start of this experiment on October 5, 2007, mussels which had been prepared for the experiment were transferred to the four exposure tanks containing full salinity seawater at a stocking rate of twenty four mussels per tank. All the mussels were fed on a daily basis and the 37 L of water in each tank were exchanged by gravity flow at a rate of between 25-35 ml min^{-1} over the course of 20 hours. This ensured that continuous water exchange occurred over the 20 hour period. On each successive day when the header tanks were filled, 3.7 L of seawater in two of the header tanks were replaced with 3.7 L of freshwater. In this way, salinity in two of the tanks was reduced from 34 ppt to 17 ppt over five days. On the day after 17 ppt was reached in two of the tanks, one half of the n=8 mussels for each of the three sampling days were removed from each tank. These were placed into two separate tanks containing 34 ppt and 17 ppt salinity seawater in the aquarium room until the following day.

After half the mussels had been removed and transferred to two tanks in the aquarium room, the copper exposures on the remaining twelve mussels were begun. The stock used to produce a seawater concentration of 100 μg Cu L^{-1} was 1.984 μg $CuCl_2 \cdot 2H_2O$ in 200 ml

dH_20. One ml of this stock was placed in the header tank and the exposure tanks for the two treatments containing 100 μg Cu L^{-1} on the first day of exposure. All the systems were allowed to exchange water until the following morning when all of the spent water was discarded and new water was added into the header tanks. On the second morning, the twelve mussels which had been removed from each tank were replaced in their original tanks and the second day of exposure proceeded. Mussels were then fed twice with thawed *Tetraselmis chuii* concentrate. Each morning at between 6:00 AM and 8:00 AM, fresh seawater was renewed in the header tanks, the spent water was removed, the mussels were checked for mortality and were then fed. After three days of exposure, a sample size of n = 8 mussels was collected and evaluated for clearance, respiration, excretion and condition. Calculations were done on a dry weight basis. This was repeated after seven and fourteen days of exposure. Sampling was done over two days based on the two day staggered process used to stock the mussels. The data were evaluated as in Section 2.10.

Experiment 2: Farm mussels exposed to 100 μg Cu L^{-1} at 17 and 34 ppt.E

Experimental design

Samples of farmed *P. canaliculus* were subject to the same four combinations of salinity and copper used in experiment 1.

1) 34 ppt & 0 μg Cu L^{-1}.
2) 17 ppt and 0 μg Cu L^{-1}.
3) 34 ppt & 100 μg Cu L^{-1}.
4) 17 ppt & 100 μg Cu L^{-1}.

These treatments were used to evaluate the effects of exposure to low salinity and copper on clearance, respiration, excretion, SfG and condition after three, seven and fourteen days of exposure.

Collection and maintenance

Farm *Perna canaliculus* mussels shipped from Pigeon Bay via same day courier were received on the 13 November, 2007. These mussels were left overnight at the SBS aquarium in a 30 L tank with gently flowing seawater. The following day, mussels were prepared for the experiment by selecting for 5.9 to 7 cm mussels which were cleaned. These were then glued to plastic plates with cyanoacrylate glue and suspended in running seawater for two days before starting of the experiment. Mussels were fed during these two days.

Experimental setup and evaluation

On November 16, 2007, the acclimation phase was started by transferring twenty-four prepared mussels to each of the four exposure tanks (described in Section 2.5) which all contained 34 ppt salinity seawater. Over the next five days, salinity was lowered from 34 ppt to 17 ppt in two of the tanks. At the end of this acclimation period, twelve of the mussels in each tank were removed and temporarily placed in two separate tanks containing 34 ppt and 17 ppt salinity seawater.

The mussels which remained in the four exposure tanks with 34 ppt and 17 ppt seawater were then exposed to 0 μg Cu L^{-1} and 100 μg Cu L^{-1} by addition of 1 ml of the stock containing 1.984 mg $CuCl_2 \cdot 2H_2O$ in 200 ml dH_2O. Mussels were then fed and the exposure system placed on flow through until the header tanks were empty. On the second day of the experiment, the 12 mussels that had been removed from each tank were replaced into their original tanks from which they were removed. Mussels were fed twice daily and water in each system was replenished with the appropriate copper spikes each day.

A total sample size of n=8 mussels was collected and evaluated for clearance, respiration and excretion rates after the exposure periods. The physiological and condition indices were calculated and expressed on a dry weight basis as in section 2.9.

7.3 Results

Experiment 1: Intertidal mussels exposed to 100 µg Cu L^{-1} at 17 and 34 ppt.

Clearance rate

Clearance rates for the controls were significantly greater than the lower rates determined for the other three treatments challenged with either copper or low salinity over fourteen days (Figure 7.1).

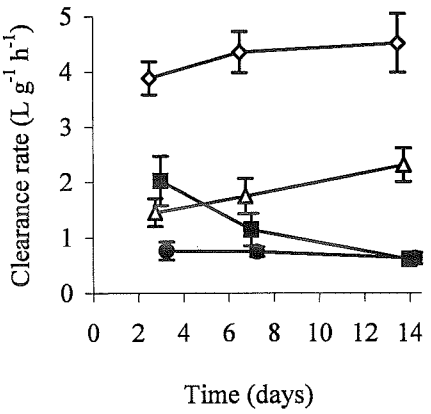


Figure 7.1: Clearance rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of variance of the entire data set with repeated measures ANOVA showed that significant effects of salinity and copper concentration were detected (Table 7.1). The salinity-copper concentration interaction was also significant. The effect of the duration of exposure was not significant.

Table 7.1: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on clearance rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.101	1	0.101	59.2	<0.001
Copper Concentration	0.203	1	0.203	119.3	<0.001
Duration of exposure	0	2	0	0	NS
Salinity*Copper concentration	0.038	1	0.038	22.3	<0.001
Salinity*Duration of exposure	0.005	2	0.003	2.2	NS
Copper Concentration*Duration of exposure	0.018	2	0.009	7.5	0.001
Salinity*Copper concentration*Duration of exposure	0.003	2	0.001	1.1	NS

Post hoc investigation of the repeated measures ANOVA using Tukey's HSD test confirmed that the clearance for the full salinity treatments was significantly greater than the clearance for the 17 ppt salinity treatments. The difference between the mussels not treated with copper and the copper exposed mussels was also confirmed to be significant.

In terms of treatment effects on individual days, differences among the four treatments were found significant for days three (ANOVA $F_{(3,28)} = 18.21$, $p < 0.001$), seven (ANOVA $F_{(3,28)} = 31.09$, $p < 0.001$) and fourteen (ANOVA $F_{(3,28)} = 41.97$, $p < 0.001$). *Post hoc* evaluation of these ANOVA results confirmed that on day three, clearance for the 17 ppt/100 µg Cu L^{-1} treatment was lower than clearance for the 34 ppt/100 µg Cu L^{-1} treatment. On day seven, all treatments except the 34 ppt/0 µg Cu L^{-1} treatment displayed similarly low clearance. On day fourteen, clearance for the 34 ppt/0 µg Cu L^{-1} treatment was significantly higher than clearance recorded for the 34 ppt/100 µg Cu L^{-1}, 17 ppt/0 µg Cu L^{-1} and 17 ppt/100 µg Cu L^{-1} treatments.

Respiration rate

Respiration rates in all the treatments were initially low but respiration in the full salinity treatments increased on day seven. Following this, there was a decline for the two full salinity treatments on day fourteen (Figure 7.2).

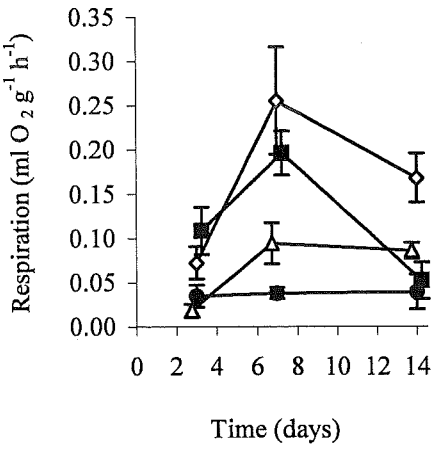


Figure 7.2: Respiration rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of the data for the three sampling days using repeated measures ANOVA showed that salinity, copper and duration of exposure all had significant effects on the respiration rates measured (Table 7.2). The interaction between salinity and copper concentration was not significant (Table 7.2).

Table 7.2: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on respiration rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0	1	0	28.94	<0.001
Copper Concentration	0	1	0	4.99	0.034
Duration of exposure	0	2	0	14.49	<0.001
Salinity*Copper concentration	0	1	0	0.26	NS
Salinity*Duration of exposure	0	2	0	6.62	0.003
Copper Concentration*Duration of exposure	0	2	0	5.83	0.005
Salinity*Copper concentration*Duration of exposure	0	2	0	0.98	NS

Post hoc evaluation of the repeated measures ANOVA with Tukey's HSD test confirmed that differences between the average respiration for the 34 ppt treatments and 17 ppt salinity treatments were significant. *Post hoc* evaluation also confirmed that difference between the copper naïve and copper exposed mussels were also significant. Finally, the *post hoc* Tukey's test also confirmed that the day seven average was significantly higher than the day three and day fourteen averages.

Analysis of data among the four treatments for each time interval using ANOVA showed significant that differences among the respiration rates of the four treatments were detected on days three (ANOVA $F_{(3,28)} = 4.03$, $p = 0.017$), seven (ANOVA $F_{(3,28)} = 7.88$, $p < 0.001$) and fourteen ($F_{(3,28)} = 8.08$, $p < 0.001$). These were confirmed by *post hoc* Tukey's HSD test.

Excretion rate

The data for day three showed that all treatments except the 17ppt/100 µg Cu L^{-1} treatment excreted at similar rates. After this, the two treatments in 34 ppt salinity maintained consistent excretion rates over the next two time intervals while excretion in the 17ppt/0 µg Cu L^{-1} and 17ppt/100 µg Cu L^{-1} treatments increased and decreased respectively (Figure 7.3).

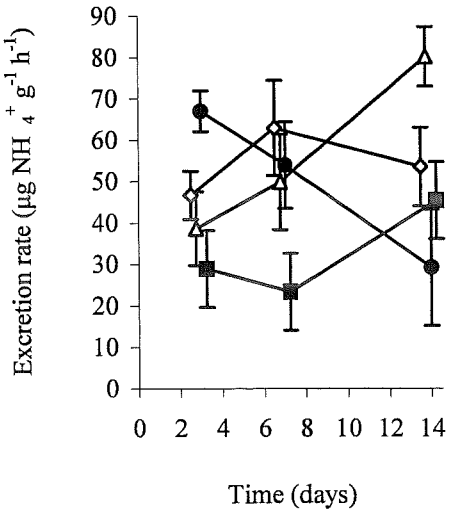


Figure 7.3: Excretion rate +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of these data using repeated measures ANOVA showed that neither salinity nor time significantly affected excretion. However, the effects of copper were significant (Table 7.3). There was no interaction between copper and salinity detected.

Table 7.3: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on excretion rate of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.783	1	0.783	2.468	NS
Copper Concentration	2.393	1	2.393	7.546	0.010
Duration of exposure	0.026	2	0.013	0.051	NS
Salinity*Copper concentration	0.71	1	0.71	2.238	NS
Salinity*Duration of exposure	2.663	2	1.331	5.301	0.008
Copper Concentration*Duration of exposure	1.868	2	0.934	3.718	0.030
Salinity*Copper concentration*Duration of exposure	0.18	2	0.09	0.358	NS

Evaluation of the repeated measures ANOVA with *post hoc* Tukey's HSD tests confirmed that the significant difference in response to copper resulted because the excretion of 55.22 μg NH_4^{+1} g^{-1} h^{-1} recorded for the mussels not exposed to copper was significantly higher than the 41.23 μg NH_4^{+1} g^{-1} h^{-1} recorded for copper exposed mussels.

Analysis of the excretion data from the four treatments after three days of exposure showed significant differences among the treatments ($F_{(3,28)} = 4.11$, $p = 0.02$). This was because the 17 ppt/100 μg Cu L^{-1} treatment excreted at rates significantly higher than the 17 ppt/0 μg Cu L^{-1} treatment. On day seven, no differences in excretion among the treatments (ANOVA $F_{(3,28)} = 2.47$, $p = 0.08$) were detected. However, on day fourteen, ANOVA detected significant differences among the treatments (ANOVA $F_{(3,28)} = 2.47$, $p = 0.015$). This result was evaluated with *post hoc* Tukey's HSD test which confirmed the difference.

Over the course of the experiment, the 34 ppt/0 µg Cu L^{-1} treatments showed consistent SfG which were higher than the SfG for all the other treatments at the three intervals (Figure 7.4).

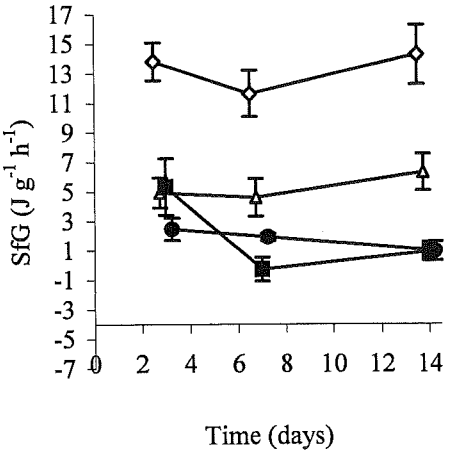


Figure 7.4: SfG +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

The SfG of the groups comprising combinations of reduced salinity and exposure to copper were stable but low for the duration of the experiment. The 34 ppt/100 µg Cu L^{-1} treatment underwent a sharp decline between days three and seven but the day three and fourteen averages were similar to the averages for the other copper or salinity stressed treatments.

Analysis of variance of the full dataset using repeated measures ANOVA showed that effects of copper, salinity and duration of exposure were significant in terms of their effects on SfG (Table 7.4). In addition, a significant interaction between copper and salinity effects was detected.

Table 7.4: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on SfG of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.437	1	0.437	19.8	<0.001
Copper Concentration	1.913	1	1.913	86.74	<0.001
Duration of exposure	0.117	2	0.058	3.94	0.025
Salinity*Copper concentration	0.457	1	0.457	20.71	<0.001
Salinity*Duration of exposure	0.071	2	0.035	2.38	NS
Copper Concentration*Duration of exposure	0.09	2	0.045	3.01	NS
Salinity*Copper concentration*Duration of exposure	0.025	2	0.012	0.83	NS

Post hoc evaluation of the repeated measures ANOVA with Tukey's HSD test confirmed the significant effects of reduced salinity, copper exposure and time. *Post hoc* evaluation of the copper-salinity interactions also confirmed pair-wise differences detected between the control (34 ppt/0 µg Cu L^{-1}) and the 17 ppt/100 µg Cu L^{-1} treatment for the duration of the experiment.

Significant differences were detected among the treatments on day three (ANOVA $F_{(3,28)} = 11.03$, $p < 0.001$), seven (ANOVA $F_{(3,28)} = 22.46$, $p < 0.001$) and fourteen (ANOVA $F_{(3,28)} = 28.87$, $p < 0.001$). Tukey's HSD test confirmed that the controls recorded significantly higher SfG than all the other treatments on the three days. On day three, the SfG of the 17 ppt/0 µg Cu L^{-1} treatment was similar to all the copper or low salinity challenged treatments. By day fourteen, SfG for the 17 ppt/0 µg Cu L^{-1} treatment was higher than the other two treatments exposed to low salinity or copper.

Condition index

The condition indices recorded in this experiment ranged between 63 and 80 with the lowest values recorded on day seven. At each time interval, the condition recorded for all four treatments were quite similar revealing no consistent effects of reduced salinity or exposure to copper (Figure 7.5).

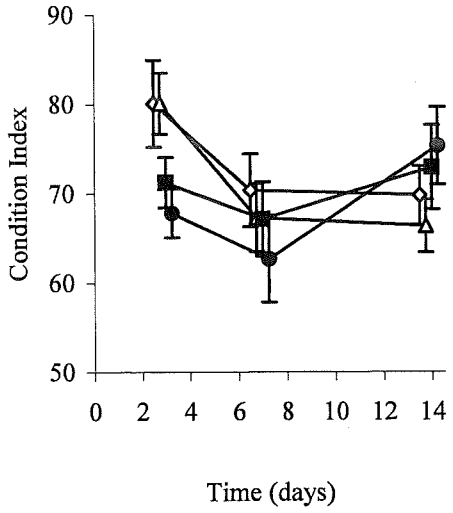


Figure 7.5: Condition index +/- SE of intertidal *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of the dataset with repeated measures ANOVA showed that no differences in condition could be attributed to either salinity or copper concentration (Table 7.5). However, there was a significant effect of duration of the exposure on condition (Table 7.5) and no interaction effects.

Table 7.5: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on condition index of intertidal *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.012	1	0.012	1.14	NS
Copper Concentration	0.024	1	0.024	2.194	NS
Duration of exposure	0.13	2	0.065	4.1	0.022
Salinity*Copper concentration	0	1	0	0	NS
Salinity*Duration of exposure	0.006	2	0.003	0.201	NS
Copper Concentration*Duration of exposure	0.123	2	0.062	3.876	0.027
Salinity*Copper concentration*Duration of exposure	0.013	2	0.006	0.408	NS

Further analysis of the condition data showed that significant differences were detected among the treatments on day three (ANOVA $F_{(3,28)} = 3.32$, $p = 0.03$). After this time, analysis of variance showed no difference among the four treatments on day seven (ANOVA $F_{(3,28)} = 0.64$, $p = 0.080$) or day fourteen (ANOVA $F_{(3,28)} = 1.01$, $p = 0.40$).

Experiment 2: Farm mussels exposed to 100 µg Cu L^{-1} at 17 and 34 ppt.

Clearance rate

Clearance for the controls was consistently high while clearance in the two treatments exposed to low salinity remained low for the duration of the experiment. On the other hand, clearance in the 34 ppt/100 µg Cu L^{-1} treatment declined steadily over the experiment (Figure 7.6).

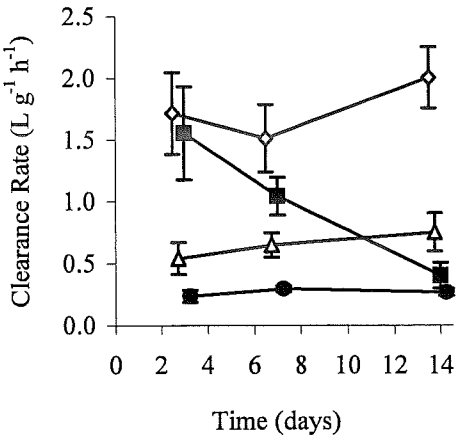


Figure 7.6: Clearance rate +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of the trends which developed over the fourteen days of the exposures using repeated measures ANOVA showed that the effects of salinity and copper were both

significant (Table 7.6). No effect of time was detected. The interactions between salinity and exposure to copper were not significant.

Table 7.6: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on clearance rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.044	1	0.044	86.28	<0.001
Copper Concentration	0.017	1	0.017	33.06	<0.001
Duration of exposure	0.001	2	<0.001	0.61	NS
Salinity*Copper concentration	0.002	1	0.002	3.1	NS
Salinity*Duration of exposure	0.003	2	0.001	1.94	NS
Copper Concentration*Duration of exposure	0.006	2	0.003	4.19	0.020
Salinity*Copper Concentration*Duration of exposure	0.004	2	0.002	2.54	NS

Post hoc tests showed that clearance for the mussels in high salinity were higher than the average for the mussels in low salinity. The significant effect of copper exposure was also confirmed by the *post hoc* test.

There were significant differences among the four treatments on days three (ANOVA $F_{(3,28)} = 8.12$, $p < 0.001$), seven (ANOVA $F_{(3,28)} = 10.12$, $p < 0.001$) and fourteen (ANOVA $F_{(3,28)} = 27.68$, $p < 0.001$). These results were confirmed with a *post hoc* Tukey's HSD which showed that both 34 ppt treatments cleared at rates higher than the 17 ppt treatments on days three and seven. The post hoc test confirmed that on day fourteen, the 34 ppt/0 µg Cu L^{-1} treatment cleared at rates higher than the other treatments.

Respiration rate

The respiration rates for all treatments were similar on day three but these diverged on day seven. Rates declined for two of the treatments on day fourteen when all treatments respired at similar rates (Figure 7.7).

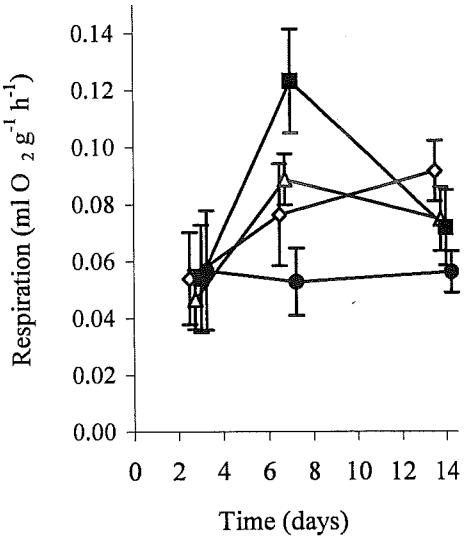


Figure 7.7: Respiration rate +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

Analysis of these trends with repeated measures ANOVA showed that salinity and time had significant effects on the respiration rates recorded. On the other hand, exposure to copper did not affect respiration (Table 7.7).

Table 7.7:Repeated measures ANOVA of effects of copper, salinity and duration of exposure on respiration rate of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	<0.001	1	<0.001	4.762	0.038
Copper Concentration	<0.001	1	<0.001	0.161	NS
Duration of exposure	<0.001	2	<0.001	4.624	0.014
Salinity*Copper concentration	<0.001	1	<0.001	2.677	NS
Salinity*Duration of exposure	<0.001	2	<0.001	0.756	NS
Copper Concentration*Duration of exposure	<0.001	2	<0.001	0.85	NS
Salinity*Copper Concentration*Duration of exposure	<0.001	2	<0.001	2.805	NS

Post hoc evaluation with Tukey's HSD test confirmed a significant difference in respiration for mussels held at 34 ppt salinity versus mussels in 17 ppt treatment. The significant effect of time was because the respiration rate on day seven was significantly higher than respiration on day three.

There were no differences among the treatments on days three (ANOVA $F_{(3,28)} = 0.07$, $p = 0.98$) or fourteen (ANOVA $F_{(3,28)} = 1.81$, $p = 0.17$). However, there were significant differences among the treatments on day seven (ANOVA $F_{(3,28)} = 3.94$, $p = 0.02$) when respiration in the 34 ppt/100 µg Cu L^{-1} treatment was greater than for the 17 ppt/100 µg Cu L^{-1} treatment.

Excretion

The excretion rates recorded for all four treatments were similar at each time interval. This was characterized by a decline on day seven after initially high rates on day three (Figure 7.8).

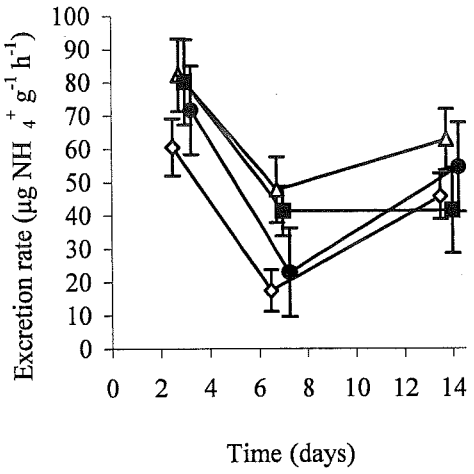


Figure 7.8: Excretion rate +/- SE of farm *P. canaliculus* exposed to 34 ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

There was no effect of either salinity or copper on excretion rate (Table 7.8). However, the effect of time was significant because of a decline between days three and seven (Table 7.8)

Both salinity and copper had significant effects on SfG (Table 7.9). No effect of time was detected but the copper-salinity interaction was significant.

Table 7.9: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on SfG of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.541	1	0.541	94.78	<0.001
Copper Concentration	0.213	1	0.213	37.31	<0.001
Duration of exposure	0.026	2	0.013	0.83	NS
Salinity*Copper concentration	0.089	1	0.089	15.59	<0.001
Salinity*Duration of exposure	0.035	2	0.018	1.13	NS
Copper Concentration*Duration of exposure	0.087	2	0.043	2.8	NS
Salinity*Copper Concentration*Duration of exposure	0.028	2	0.014	0.89	NS

Post hoc test confirmed that both the low salinity and copper treatments had significantly lower SfG. Analysis of the significant salinity-copper interaction showed that this was because the mussels in high salinity reacted more dramatically to exposure to copper than mussels in low salinity.

There were significant differences among the treatments on day three (ANOVA $F_{(3,28)} = 5.64$, $p = 0.003$) because values in 34 ppt and 17 ppt were different. This was not repeated on day seven because only the 34 ppt/0 μg Cu L^{-1} treatment was different from the all the other treatments. On day fourteen, there were differences (ANOVA $F_{(3,28)} = 6.41$, $p = 0.0019$) due to the 34 ppt/0 μg Cu L^{-1} treatment being different to all other treatments.

Condition index

Over the fourteen day course of the experiment, the mean condition of all the treatments except the 17 ppt/ 0 μg Cu L^{-1} remained relatively constant (Figure 7.10). The consistent decline in condition of the 17 ppt/ 0 μg Cu L^{-1} treatment was the only odd trend over the fourteen days of the experiment. The standard errors were substantial for all the treatments and there was considerable overlap in the data at all three time intervals (Figure 7.10).

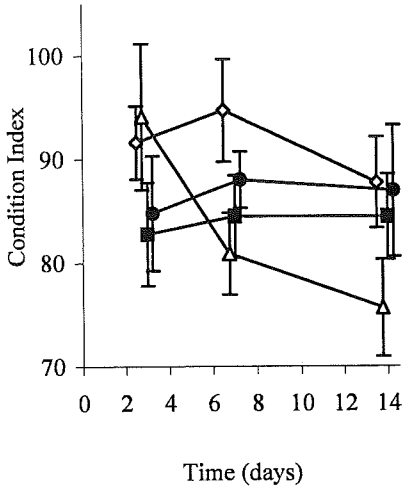


Figure 7.10: Condition index +/- SE of farm *P. canaliculus* exposed to 34ppt/0 µg Cu L^{-1} (◇), 34ppt/100 µg Cu L^{-1}(■), 17ppt/0µg Cu L^{-1}(△) and 17ppt/100 µg Cu L^{-1} (●) after three, seven and fourteen days.

When the data were analysed using repeated measures ANOVA, neither salinity, copper concentration nor time were shown to have significantly affected condition in these farm mussels (Table 7.10). Also, there were no interaction effects between salinity and copper concentration.

Table 7.10: Repeated measures ANOVA of effects of copper, salinity and duration of exposure on condition index of farm *P. canaliculus*.

Source of Variation	SS	df	MS	F	p
Salinity	0.018	1	0.018	0.957	NS
Copper Concentration	0.009	1	0.009	0.5	NS
Duration of exposure	0.033	2	0.017	0.974	NS
Salinity*Copper concentration	0.063	1	0.063	3.431	NS
Salinity*Duration of exposure	0.022	2	0.011	0.657	NS
Copper Concentration*Duration of exposure	0.063	2	0.031	1.841	NS
Salinity*Copper Concentration*Duration of exposure	0.029	2	0.015	0.856	NS

No significant differences were detected among the treatments on day three (ANOVA $F_{(3,28)} = 1.00$, $p = 0.41$), seven ($F_{(3,28)} = 1.87$, $p = 0.16$) or fourteen (ANOVA $F_{(3,28)} = 1.24$, $p = 0.31$).

7.4 Discussion

The main conclusion which can be drawn from the results of these experiments is that both farm and field mussels were severely affected by 100 μg Cu L^{-1}. This was manifested as lowered clearance, respiration, excretion and SfG for intertidal mussels but only lowered clearance and SfG for farm mussels. The condition of neither farm nor intertidal mussels was affected by copper. These results concur with previous research which has shown that copper affected functioning of the gills of the mussels (Redpath and Davenport, 1998). Whether farm or intertidal mussels are more vulnerable to copper or salinity stress has never been fully studied. The experiments in this chapter showed that the effects of copper were seen in a wider range of physiological processes in the intertidal mussels. On the other hand, the only physiological processes affected in the farm mussels involved inhibition of the particle clearance system.

The results also show that salinity affected the clearance, respiration and SfG of the farm as well as intertidal mussels. Salinity did not cause significantly different excretion rates in either the farm or the intertidal mussels. The current results are therefore consistent with previous research which has shown that copper affects diverse physiological processes in sensitive bivalves (Munari and Mistri, 2007).

In addition to the main effects of copper exposure, salinity and duration of the exposure, a number of disparities for specific treatments and treatment groups were noted between farm and intertidal mussels.

Clearance rate

Clearance rates for farm mussels were generally less than one-half the clearance for the intertidal mussels (Table 7.11). Lower clearance rates for mid shore specimens of *Perna*

canaliculus (Marsden and Weatherhead, 1999) and *Choromytilus meridionalis* (Griffiths, 1981) compared to clearance of high shore specimens have also been recorded previously.

Table 7.11: Mean clearance rates (L g^{-1} hr^{-1}) of farm and intertidal mussels exposed to combinations of full and 17 ppt salinity and 0 and 100 µg Cu L^{-1}

Treatment	Farm	Intertidal
34 ppt/ 0 µg Cu L^{-1}	1.74 ± 0.28	4.25 ±0.40
17 ppt/ 0 µg Cu L^{-1}	0.65 ± 0.13	1.84 ± 0.29
34 ppt/ 100 µg Cu L^{-1}	1.00 ± 0.21	1.26 ± 0.27
17 ppt/ 100 µg Cu L^{-1}	0.27 ± 0.03	0.71 ± 0.12

A second important difference was that clearance for intertidal mussels in the 34 ppt/100 µg Cu L^{-1} treatment declined over the fourteen days of exposure. This decline was delayed until after day three for the farm mussels. This suggests that the farm mussels were initially able to avoid exposure or were able to withstand the exposure for some time. The ability of some mussels to seal the valves and isolate themselves from contaminated seawater has been demonstrated for naturally occurring *Mytilus edulis* (Manley, 1983). Avoidance of the contaminated water is therefore a more likely explanation because exposure to copper at concentrations as low as 86 µg Cu L^{-1} has been shown to be lethal to mussels (Krishnakumar *et al.*, 1987). In addition, farm *P. canaliculus* have been shown to be able to reduce their clearance rates in response to high turbidity (Hawkins *et al.*, 1999). If the farm mussels in the present study were indeed partially isolating themselves from the contaminated medium by decreasing their filtration, a behavioral response may have played an important part of the physiological responses to copper measured for farm mussels (Mouabad *et al.*, 2001).

Clearance rates of the farm and field mussels in the 17 ppt/0 µg Cu L^{-1} treatment were also different. In the case of field mussels, clearance increased between days three and

fourteen. However, clearance remained the same for the farm mussels. This suggested that the intertidal mussels were acclimating to low salinity better than the farm mussels.

Respiration rate

Respiration was generally found to be lower for the farm mussels compared to the intertidal mussels (Table 7.12). This was most evident for the full salinity controls but was also seen in the 34 ppt/100 μg Cu L^{-1} treatments for both experiments.

Table 7.12: Mean respiration rates (ml O_2 g^{-1} hr^{-1}) of farm and intertidal mussels exposed to combinations of full and 17 ppt salinity and 0 and 100 μg Cu L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 μg Cu L^{-1}	0.07 ± 0.01	0.17 ± 0.04
17 ppt/ 0 μg Cu L^{-1}	0.07 ± 0.01	0.07 ± 0.01
34 ppt/ 100 μg Cu L^{-1}	0.08 ± 0.02	0.12 ± 0.02
17 ppt/ 100 μg Cu L^{-1}	0.06 ± 0.01	0.04 ± 0.01

Exposure to reduced salinity also caused a significant decline in respiration in both the copper exposed and copper naïve intertidal mussels. This decline in respiration due to reduced salinity was not seen in the farm mussels. Decreased respiration from 0.92 ± 0.06 ml O_2 g^{-1} hr^{-1} to 0.46 ± 0.04 ml O_2 g^{-1} hr^{-1} as a result of transfer of mussels collected from North Sea sites of 28.8 ppt salinity to 16.6 ppt has been seen in *Mytilus edulis* (Tedengren and Kautsky, 1987). Along with the decline in respiration due to reduced salinity, a significant decline in respiration due to copper was detected for the intertidal mussels. The depression in respiration rate of intertidal mussels detected in the current experiments was unlike results obtained for the clams *Tapes philippinarum* and *Ruditapes decussatus* which recorded an increase of 120% in respiration rates after two days of exposure and an increase of 150% after 20 days of exposure to 10 μg Cu L^{-1} (Sobral and Widdows, 1997b). The difference in respiration between the present study and the study on the clams may be because the 100 μg Cu L^{-1} exposure used in the present experiments may have caused

irreversible gill damage along with a disruption in the capacity to utilize dissolved oxygen in the seawater media (Anandraj *et al.*, 2002).

Excretion rate

In the present study, excretion of either farm or intertidal mussels was not affected by reduced salinity (Table 7.13). Previous studies have shown that excretion in the mussel *Choromytilus chorus* increased from 8.78 μg NH_4^+ g^{-1} h^{-1} to 16.22 μg NH_4^+ g^{-1} h^{-1} between 30 ppt and 18 ppt. However, at a salinity of 15 ppt, excretion was 3.49 μg NH_4^+ g^{-1} h^{-1} in this mussel (Navarro, 1988). Between salinities of 24 ppt and 18 ppt, excretion in the scallop *Argopecten purpuratus*, declined from 41.9 μg NH_4^+ g^{-1} h^{-1} to 19.7 μg NH_4^+ g^{-1} h^{-1}. This low excretion was probably because the mussels were approaching the limit of their capacity to adapt to low salinity.

In the present study, exposure to copper did not affect excretion in farm mussels but produced significantly reduced excretion in intertidal mussels (Table 7.13).

Table 7.13: Mean excretion rates (μg NH_4^+ g^{-1} hr^{-1}) of farm and intertidal mussels exposed to combinations of full and 17 ppt salinity and 0 and 100 μg Cu L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 μg Cu L^{-1}	41.17 ± 7.23	54.31 ± 9.92
17 ppt/ 0 μg Cu L^{-1}	64.14 ± 9.96	56.14 ± 9.19
34 ppt/ 100 μg Cu L^{-1}	54.21 ± 11.02	49.98 ± 9.80
17 ppt/ 100 μg Cu L^{-1}	49.61 ± 10.13	32.48 ± 13.87

The decline in excretion recorded for the intertidal *P. canaliculus* exposed to copper was similar to reduced excretion documented for *Sesarma quadratum* crabs exposed to 2,800 μg Cu L^{-1} and 9,300 μg Cu L^{-1} which remained low over twenty-one days of exposure (Valarmathi and Azariaha, 2002). However, the decline in excretion shown by the

intertidal mussels in the current study was not seen in *Mytilus edulis* exposed to 20 μg Cu L^{-1} (Moore *et al.*, 1984). The decline in excretion seen in the intertidal mussels in the current experiments was also in contrast to increased excretion by *Perna viridis* from 7.8 ± 2.8 μg NH_4^+ g^{-1} h^{-1} to 123.9 ± 75.8 μg NH_4^+ g^{-1} h^{-1} after two weeks of exposure to 25 μg Cu L^{-1} (Krishnakumar *et al.*, 1990).

The results compiled in these experiments showed that reduced salinity had no effect on excretion in either farm or intertidal mussels but that copper reduced excretion for intertidal mussels only.

Scope for Growth

SfG values calculated in the present study were much higher for the intertidal mussels compared to the farm mussels (Table 7.14). This may have been due to allometric scaling because the dry weights and condition were higher for the farm mussels (Griffiths, 1979). This declining function between body weight and SfG may have developed because increased feeding in intertidal mussels exceeding 1.5 g dry weight did not compensate for reduced immersion time during the low tide (Marsden and Weatherhead, 1999). The higher SfG for intertidal mussels could also have been due to phenotypic adaptation because higher feeding rates but lower condition of intertidal versus subtidal mussels have been documented for *P. canaliculus* (Marsden and Weatherhead, 1999; Petes *et al.*, 2007).

The results of the experiments show that reduced salinity caused a 99% decline in SfG for the farm mussels but only caused a 60% decline in SfG for the intertidal mussels. On the other hand, exposure to copper caused a 70% decline in SfG for farm mussels and a 85% percent decline for the intertidal mussels (Table 7.14). This is similar to the results from chapter four when exposure resulted in a 91% decline in SfG on exposure to 100 μg Cu L^{-1}. (Figure 4.14). This was also similar to the case of *Ruditapes decussates*, in which SfG decline from 16.1 J g^{-1} h^{-1} to 4.9 J g^{-1} h^{-1} after fourteen days of exposure to 10 μg Cu L^{-1} (Sobral and Widdows, 1997b).

Table 7.14: Mean SfG (J g^{-1} L^{-1}) of farm and intertidal mussels exposed to combinations of full and 17 ppt salinity and 0 and 100 µg Cu L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 µg Cu L^{-1}	4.91 ± 1.12	13.21 ± 1.62
17 ppt/ 0 µg Cu L^{-1}	0.05 ± 0.45	5.25 ± 1.18
34 ppt/ 100 µg Cu L^{-1}	1.47 ± 1.07	1.97 ± 1.11
17 ppt/ 100 µg Cu L^{-1}	-0.63 ± 0.27	1.75 ± 0.55

When copper exposure was combined with exposure to low salinity there was an immediate and severe effect comprised of negative SfG for farm mussels on day three (Figure 7.9). This critical effect of salinity may be because mytilid mussels are osmoconformers (Taylor and Anstiss, 1999). These bivalves are not able to regulate the volume of their cells but they rely on strategies such as valve closure and hydrolysis of mantle tissue to liberate free amino acids which they use to osmoregulate (Bayne *et al.*, 1976a; Berger and Kharazova, 1997). In addition to these physiological responses, mussels have shown adaptation in the form of phenotypes which are more fit to specific environments (Tedengren *et al.*, 1990). The results in the current experiments suggest that intertidal mussels possessed better adaptation to low salinity than the farm specimens. The nature of this adaptation is unknown in *P. canaliculus* but such phenotypic adaptation may include differences in rate of salt loss from the mantle in mussels exposed to low salinity (Berger and Kharazova, 1997). However, this was not tested in the current experiments. On the other hand, excretion by the intertidal mussels exposed to 17 ppt alone was numerically the highest on day fourteen (Figure 7.3). This suggests mobilization of free amino acids for the purpose of osmoregulation in the intertidal mussels (Shumway and Youngson, 1979). Conversely, the low SfG for the farm mussels in the 17 ppt treatment suggests a comparatively limited capacity to adapt to low salinity.

When exposed to copper, SfG of both farm and intertidal mussels was severely affected by 100 µg Cu L^{-1}. The decline in SfG in full salinity was more pronounced for the intertidal mussels. However, at low salinity, the negative SfG for the farm mussels exposed to copper suggests acute effects.

Condition index

The condition of farm mussels was consistently higher than that of intertidal mussels (Table 7.15). The higher condition in the farm mussels may indicate larger tissue reserves on which to rely compared to intertidal mussels. Neither salinity nor exposure to copper affected the condition of the farm mussels. Salinity and copper did not affect intertidal mussels either (Tables 7.5 & 7.10) but there was a significant effect of time on the condition of the intertidal mussels (Table 7.5). The reason for this decline in all the treatments is not known but it could have been caused by lack of feed (Marsden, 2004).

Table 7.15: Mean condition index of farm and intertidal mussels exposed to combinations of full and 17 ppt salinity and 0 and 100 µg Cu L^{-1}.

Treatment	Farm	Intertidal
34 ppt/ 0 µg Cu L^{-1}	91.36 ± 4.28	73.39 ± 4.07
17 ppt/ 0 µg Cu L^{-1}	83.55 ± 5.23	71.23 ± 3.40
34 ppt/ 100 µg Cu L^{-1}	83.90 ± 4.34	70.42 ± 3.90
17 ppt/ 100 µg Cu L^{-1}	86.58 ± 4.86	68.58 ± 3.98

Interactions

In addition to the significant effects of salinity and copper on SfG, the interaction between salinity and copper was significant for the SfG for farm and intertidal mussels. This interaction arose because, for both farm and intertidal mussels in low salinity, exposure to copper did not significantly affect the SfG. At normal salinity, the effects of copper were significant. This indicates that copper was interfering with the ability to maintain proper functions to a greater degree in full salinity. However, these results failed to show synergistic effects between salinity and the metals, unlike the effects detected between copper and phenanthrene (Moore *et al.*, 1984). The current results are similar to results which showed that exposure of *Trochus maculates* to 20 µg Cu L^{-1} in reduced salinity

caused no synergies between salinity and copper to be detected because low salinity alone caused numerically lower SfG than the combination of low salinity and copper (Elfwing and Tedengren, 2002b)

Conclusions

This research confirmed that reduced salinity was a key factor contributing to stres in farm as well as intertidal *P. canaliculus* exposed to copper (Elfwing. and Tedengren, 2002; Prevodnik *et al.*, 2007; Hamer *et al.*, 2008). Previous studies have shown that salinity affects clearance, respiration and excretion in bivalves (Navarro, 1988).

In addition to the systematic effect of salinity, these results showed that the effects of copper were different for farm and intertidal mussels because copper affected only the clearance function in farm mussels (Redpath and Davenport, 1998) whereas it affected clearance, respiration and excretory (Grosell *et al.*, 2007) functions in the intertidal specimens. The latter effects could have resulted from impairment of membranes (Taylor and Anstiss, 1999) other than the gills in the intertidal mussels. This could have occurred because the intertidal mussels maintained significantly higher clearance rates than the farm specimens. These high clearance rates may have physically exposed more mantle tissue to dissolved copper in the case of the intertidal mussels.

The results of these comparative experiments provided evidence that there was a greater decline in SfG of intertidal mussels exposed to copper in full salinity compared to the farm mussels exposed to the same treatment. The research also showed that more physiological systems were affected by copper in the intertidal than in the farm specimens. However, only the farm mussels showed negative SfG. This suggests that the physiological data which could be gathered from the naturally occurring intertidal mussels exposed to effluents containing copper would be better than data from farm mussels. This is because SfG of intertidal mussels allowed factors such as salinity to be detected. Apart from factors such as salinity, temperature and feed, the time of year could play an important role in the physiology of bivalves. Together, these factors contribute to the physiological responses of bivalve filter feeders to various toxins and need to be analysed as a whole in order to validate the best practices available for use of bivalves such as *P. canaliculus* as biomonitors (Widdows *et al.*, 1984).

Chapter 8 General Discussion

8.1 General trends

Increases in concentration of cadmium and copper in aquatic environments can cause long term ecosystem and human health effects which need to be understood by society (Nriagu, 1990; Reiley, 2007). Programmes like the Global Mussel Watch have documented the extent of the problem at many locations between the 1960s and the present (O'Connor and Lauenstein, 2006). However, information which links the structure of ecosystems with measurable sub-organismal changes in keystone species is lacking (Knap *et al.*, 2002). The SfG is one method which has allowed for measurements on individual animals to be used to indicate the state of ecosystems (Crowe *et al.*, 2004). This thesis investigated the effects of copper and cadmium on SfG of naturally occurring and farmed *P. canaliculus* mussels in normal and reduced salinity.

The aim of the study was to determine if there were any differences in severity of the toxic effects of copper or cadmium on farm or intertidal mussels. The effects of reduced salinity on both types of mussels were also investigated. Additionally, mussels were exposed to combinations of reduced salinity and the trace metals in order to determine if the stressors were acting synergistically or antagonistically. These experiments investigated the effects of copper because it is widely used as an anti-foulant and has caused mass mortalities, tumour-like growth and abnormal embryo development of mussels and fish (Martin *et al.*, 1977; Schiff *et al.*, 2007; USEPA, 2008). This study also investigated the effects of

cadmium because few studies have been able to show physiological effects of this metal on shellfish (Anderlini, 1992). Prior to the current use of copper as the antifouling chemical of choice in the marine industry, TbT was used in the global shipping and pleasure boat industries. TbT caused local extinctions of shellfish in the Netherlands (Cadee *et al.*, 1995), Japan (Horiguchi *et al.*, 2006) and the UK (Bryan *et al.*, 1986) and was subsequently banned by many governments including New Zealand in 1989 (Smith and McVeagh, 1991).

Substantial declines in populations of naturally occurring *P. canaliculus* (Hauraki Maori Trust Board, 1999; Dawber, 2003) and fourteen other molluscs have occurred in the Hauraki Gulf between 1937 and 1990 (Moore *et al.*, 2004a). The decline in mussels in the Hauraki Gulf was not because of trace metal pollution. However, elevated levels of copper and tributyltin have been recognized in fish and shellfish in New Zealand (Kennedy, 1986; Stoffers *et al.*, 1986; Smith and McVeagh, 1991; Winchester, 1998; Perera, 2004). Additionally, it is recognised that there is a need to remain vigilant in the protection of *P. canaliculus* resources because current and future anthropogenic pressures relating to climate change (Petes, 2007) and ocean acidification could affect growth of intertidal specimens which are believed to be genetically distinct from subtidal specimens (Sin *et al.*, 1990).

A useful tool to monitor the habitats which *P. canaliculus* occupy is SfG (Tasman *et al.*, 2004). Previous studies on the SfG of mussels from coastal and offshore locations in the North Sea have shown that differences in the energetic balance of *Mytilus edulis* were related to contaminant concentrations on a spatial scale exceeding 1,000 km (Widdows *et al.*, 1995). The SfG is a useful method to assess the environmental quality of a site without measuring the actual growth rate of mussels at the site (Bayne, 1998). SfG has indicated the impacts of natural productivity (Gardner and Thompson, 2001; Helson and Gardner, 2007) and pollutants (Anderlini, 1992) on mussel physiology at aquaculture and outfall sites in New Zealand. However, in a number of cases, physiological indices including SfG and clearance of mussels were not affected by important pollutants including cadmium and crude oil (Poulsen *et al.*, 1982; Anderlini, 1992; Redpath and Davenport, 1998; Thomas *et al.*, 1999).

The current study was developed to determine how various factors affect the SfG of mussels. Specifically, the study investigated differences in SfG of farm and intertidal *P.*

canaliculus exposed to copper and cadmium at full salinity and ½ salinity. The early experiments failed to show repeatable results for the controls and failed to provide clear evidence of a depression of SfG due to 1 μg Cu L^{-1}, 10 μg Cu L^{-1} or 100 μg Cu L^{-1}. The reason may be related to the fact that *Perna canaliculus* is a filter feeder with one of the highest clearance rates known (Hawkins *et al.*, 1999). The clearance in *P. canaliculus* is also very sensitive to environmental cues (Hawkins *et al.*, 1999). This recognition led to the development of a unique experimental method to determine SfG using handling of individual mussels which were kept in mesocosms replicating their natural environments.

8.2 Energetics of the controls

Clearance rate

Clearance rate is one of the most studied physiological measurements conducted on bivalves. It is recognized as an important physiological measure for mussels because changes in this physiological measurement can occur within two hours of contact with sub-lethal concentrations of trace metals such as copper and mercury (Mouabad *et al.*, 2001; Toro *et al.*, 2003). In addition, the equipment needed to quantify the rate of clearance of chlorophyll in water is commonly available in most biological laboratories (Resgalla *et al.*, 2007b; Elliott *et al.*, 2008). However, there is still some discussion about the best method to determine clearance because some methods are thought to underestimated the value (Riisgård, 2001; Widdows, 2001). One factor which can affect clearance rate is the feeding status of the mussels leading up to the evaluations (Navarro, 1989). Low clearance rates have been recorded for the mussel *Modiolus modiolus* when seston quantity was low (Navarro, 1989). Differences in the hydrodynamics of tanks used to conduct the clearance procedures in flow through systems are also important (Filgueira *et al.*, 2006; Elliott *et al.*, 2008). When the velocity of water in flow through chambers was high, the filtration rate recorded was reduced because there was proportionally less draw-down than the draw-down recorded when the chambers were under low flow rates. On the other hand, when the flow rates were low, the clearance rates recorded were higher (Elliott *et al.*, 2008).

In the experiments described in the current work on *Perna canaliculus*, clearance recorded for the intertidal controls as well as copper exposed treatments were also low in the first three copper exposure experiments in chapter four. The highest value of clearance was

1.89 L g^{-1} h^{-1} for the controls (Table 8.1). These low values were recognized and the exposure and clearance evaluation procedures were modified. With the modifications, clearance in the controls was almost doubled. These rates were slightly higher than clearance recorded for the same species in Wellington harbour (Gardner, 2000; Helson and Gardner, 2007) and Christchurch (Marsden and Shumway, 1992a) (Table 8.1).

Table 8.1: Clearance rates determined for *P. canaliculus* and other mussel species.

Species	T (°C)	Clearance rate (L g^{-1} h^{-1})	Reference
P. canaliculus	12.5-18.3	3.34	(Helson and Gardner, 2007)
P. canaliculus	10.0-15.9	3.3	(Gardner, 2002)
P. canaliculus	10.4-15.9	3.4-4.6	(Gardner, 2000)
P. canaliculus	15	2.0-4.6	(Marsden and Shumway, 1992b)
P. canaliculus	15	1.1-3.7	(Weatherhead, 1993)
P. canaliculus	15	2.3-8.8	(Waite, 1989)
P. canaliculus	15-19.5	3.33	(Gardner and Thompson, 2001)
Mytilus. galloprovincialis	10.0-15.9	4.0	(Gardner, 2002)
Mytilus californianus (starved)	13	1.64	(Bayne *et al.*, 1976b)
Mytilus californianus (fed)	13	1.04	(Bayne *et al.*, 1976b)
Mytilus edulis	11-20	7.6	(Martin *et al.*, 1984)
Choromytilus chorus	12	1.56	(Navarro, 1988)
Mytilus. edulis	0-15	1.5-2.0	(Thompson, 1984)
Mytilus edulis	10	4.2	(Riisgård *et al.*, 2003)
Mytilus edulis	7-21	1.3-2.6	(Bayne and Widdows, 1978)
Mytilus galloprovincialis		3.1-4.8	(Filgueira *et al.*, 2006)
Choromytilus meridionalis (subtidal)	12	0.94	(Griffiths, 1980b)
Choromytilus meridionalis (intertidal)	12	1.16	(Griffiths, 1980b)
P. canaliculus	15	4.07-5.42	This study (Chapter 4-7)
P. canaliculus	15	1.01-1.89	This study (Chapter 3)

The rates in the present study were slightly higher than clearance recorded for *Mytilus edulis* in Canada (Thompson, 1984) and Europe (Bayne and Widdows, 1978) but were substantially higher than clearance of *Choromytilus meridionalis* in South Africa (Griffiths, 1980a). In contrast to the slightly elevated clearance in the intertidal mussels, clearance by the farm mussels averaged 2.18 L g^{-1} h^{-1} which was slightly below values seen in the Wellington and Canterbury regions (Table 8.1). The lower clearance rate recorded for the farm mussels may be related to a mechanism which these mussels possess to decrease clearance in order to prevent overloading of the filtration mechanism in the gills (Scholten and Smaal, 1998; Hawkins *et al.*, 1999). In the case of *Perna canaliculus*, feeding depression has been noted for farmed specimens offered rations in excess of 2 µg, chlorophyll L^{-1} (Hawkins *et al.*, 1999).

It is not know whether differences in the capacity to decrease clearance is shared between farm and intertidal species or specimens. However, an opposite feeding behavior has been shown in intertidal *Geukensia demissa*. These intertidal mussels showed significantly higher clearance rates for specimens which were emersed for 75% of a twelve hour tidal cycle (Charles and Newell, 1997). This could have accounted for the greater clearance rates noted for the intertidal specimens in the current experiments.

In addition to more realistic clearance rates for the controls, significant effects of 100 µg Cu L^{-1} on clearance rates were detected on intertidal mussels in the last copper exposure experiment in chapter four (Figure 4.11). Subsequent to this experiment, the rates of clearance measured for the intertidal mussel controls in this research averaged from 4.83 L g^{-1} h^{-1} for the experiments in chapters five, six and seven of the current study (Table 8.1). The difference between the low clearance in the first three copper exposure experiments in chapter four and later values in the thesis were likely to have resulted because of reduced handling stress and improved nutrition in the latter experiments. These two issues were not thoroughly addressed in the earlier experiments.

Subsequently, clearance rates of the intertidal controls were similar in the preliminary cadmium experiments in chapter five and the low salinity cadmium and copper experiments of chapters six and seven. An important factor in these results was probably the addition of organic particles in the form of preserved *Tetraselmis chuii* cells into the

exposure system to served as stimuli for filtration by the bivalves (Bayne *et al.*, 1976a). In addition to the presence of these particles, the stimulation of heterotrophic bacteria by addition of artificial feed, along with improved aeration and better water flow from the protein skimmers (Figure 2.8) cannot be discounted as important food for the mussels (Kaspar *et al.*, 1985; Kautsky and Evans, 1987). Heterotrophs also play important roles in the degradation of wastes and metabolites in mesocosm studies (Laursen *et al.*, 2002).

Respiration rate

In the present study, the respiration rates recorded for control intertidal mussels collected in June and September 2006 were similar to respiration of specimens from the Canterbury region (Marsden and Shumway, 1992a; Marsden and Weatherhead, 1999; James *et al.*, 2001) but slightly lower than mussels from the Wellington region (Gardner, 2000; Helson and Gardner, 2007) (Table 8.2). The rates were also generally within the range of *Mytilus edulis* from sub-arctic sites (Thompson, 1984) and *Mytilus californianus* (Bayne *et al.*, 1976b) fed with a mixture of *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Dunaliella* sp. However, the rates in the current experiments were lower than rates recorded for *Choromytilus. meridionalis* in South Africa (Griffiths, 1980b) and *M. edulis* in Sweden (Tedengren *et al.*, 1990).

The initial experiments showed that the maintenance regime applied to mussels affected their metabolic rates. The first factor which was shown to affect the respiration rate of mussels held under prolonged laboratory conditions was the practice of using only naturally occurring seston in the seawater to maintain laboratory held mussels. The early experiments showed that a decline in respiration rate for mussels receiving no supplemental algal feed would occur within two weeks of transfer of the mussels to the laboratory (Section 3.3, Experiments 1 & 2). A similar decline in respiration of *M. edulis* was noted for mussels starved between 16 and 23 days (Bayne and Thompson, 1970; Bayne *et al.*, 1976b).

Table 8.2: Respiration rates determined for *Perna canaliculus* and other mussel species.

Species	T (°C)	Respiration rate (ml O_2 g^{-1} h^{-1})	Reference
P. canaliculus	12.5-18.3	0.256	(Helson and Gardner, 2007)
P. canaliculus	10.4-15.9	0.45	(Gardner, 2000)
P. canaliculus	15	0.26	(Marsden and Shumway, 1992b)
P. canaliculus	15	0.17-0.26	(Marsden and Weatherhead, 1998)
Choromytilus. meridionalis	12	0.43	(Griffiths, 1980b)
Mytilus edulis	0.15	0.10-0.44	(Thompson, 1984)
Mytilus edulis	9-16.5	0.079-0.356	(Bayne *et al.*, 1987)
Mytilus galloprovincialis	14.3-15	0.53-0.70	(Babarro *et al.*, 2000b)
P. canaliculus	11-17.4	0.189-0.667	(James *et al.*, 2001)
P. canaliculus	15-19.5	0.45	(Gardner and Thompson, 2001)
Mytilus edulis	12	0.95	(Tedengren *et al.*, 1990)
Mytilus californianus (unfed)	13	0.136	(Bayne *et al.*, 1976b)
Mytilus californianus (fed)	13	0.232	(Bayne *et al.*, 1976b)
Mytilus edulis	11-20	0.6	(Martin *et al.*, 1984)
Mytilus edulis	15	0.20	(Widdows and Shick, 1985)
P. canaliculus	15	0.15 ± 02	Intertidal mussels before feeding (Chapter 3)
P. canaliculus	15	1.03 ± 0.16	Intertidal mussels 1 hr after feeding (Chapter 3)
P. canaliculus	15	0.21 ± 0.08	Intertidal mussels kept for 2 months with feed in laboratory (Chapter 3)
P. canaliculus	15	0.09 ± 0.05	Intertidal mussels kept for 2 months without feed in laboratory (Chapter 3)
P. canaliculus	15	0.22 ± 0.10	Recently harvested intertidal mussels (Chapter 3)
P. canaliculus	15	0.09 ± 0.00	Intertidal mussels kept for 2 weeks in laboratory (Chapter 3)
P. canaliculus	15	0.14	Intertidal mussels: Chapter 4
P. canaliculus	15	0.16	Intertidal mussels: Chapter 5
P. canaliculus	15	0.11	Farm mussels: Chapters 6&7
P. canaliculus	15	0.18	Intertidal mussels: Chapters 6&7

This pattern of reduced respiration in unfed mussels was again detected when a difference in respiration rate was detected between fed and unfed mussels after two months (Section 3.3, Experiment 5). For the unfed mussels, respiration rate was unstable over a 48 hour period. This erratic behavior could have been the result of intermittent opening and closing versus a more continuous filtering activity. Such behaviour corresponds to an energy saving strategy in mussels receiving only minimal access to a food substrate (Riisgård and Randløv, 1981).

The second important finding was that there was an increase in respiration immediately after feeding of the mussels (Figure 3.11). This elevated respiration comprised the routine metabolism as well as the specific dynamic action. This spike in oxygen consumption could also have indicated active metabolism (Thompson and Bayne, 1972). In addition to high metabolism after feeding, the high rates could have included additional oxygen consumption involved with foot movements and the eventual formation of byssus and attachment of the mussels unto the sides of respirometers. This issue of mussels tending to form byssal attachments within the period of evaluation of clearance, respiration and excretion was addressed after low and highly variable clearance and respiration rates were recorded for the controls in the first three copper exposure experiments in chapter 4.

The attention paid to byssus threads was in light of the fact that it takes two minutes for the mussel foot to secrete a byssal plaque (Nishida *et al.*, 2003) and less than 30 minutes for a mussel to become securely attached (Carton *et al.*, 2007). Byssal attachment may account for 8% of the monthly energy requirement of mussels but there is little documentation on the effect of foot movement and other attachment related processes on clearance rate of mussels (Hawkins and Bayne, 1985). However, Griffiths (Griffiths, 1980b) cites Winter (Winter, 1969) who suggested that differences were noted in the clearance rates of attached versus unattached mussels. Numerous recent studies have subsequently used attached mussels to conduct physiological experiments (Waite, 1989; Bruner *et al.*, 1994; Rajagopal *et al.*, 2005; Elliott *et al.*, 2008). The work in this thesis used this approach so byssal attachments made by the mussels did not have to be severed during the experiment.

After adjustments to the handling and maintenance of mussels were made, respiration rates obtained for the controls in the last copper exposure experiment in chapter four were low compared to previous work on *P. canaliculus* (Table 8.2). One of the reasons for low respiration rates for the controls in chapters six and seven may have been that settling

times were reduced from an overnight period in the initial respirometry experiments to two hours in the copper or cadmium exposure experiments. This shorter settling period was implemented in order to be able to conduct clearance, respiration and excretion experiments on sixteen mussels in one day.

In addition to differences in respiration noted in the experiments in chapter three and the subsequent experiments, consistent differences in respiration were noted between the farm and the intertidal mussels in chapters five, six and seven. For the intertidal mussels, the respiration rate averaged 0.18 $\pm$ 0.03 ml O_2 g^{-1} h^{-1} while respiration of the farm mussels averaged 0.11 $\pm$ 0.02 ml O_2 g^{-1} h^{-1} (Table 8.2). These rates were lower than the respiration rates recorded for rope and intertidal *M. gallporovincialis* (Babarro *et al.*, 2000b). The *M. gallprovincialis* intertidal specimens showed lower respiration than farm specimens upon transfer to a culture raft but between 22-110 days of culture, the intertidal mussels recorded higher respiration rates (Babarro *et al.*, 2000b). In the present study, the higher respiration of the intertidal mussels may have also been a result of allometric scaling because the average dry tissue weight of the farm mussels was 1.96 $\pm$ 0.17 g compared to 0.99 $\pm$ 0.19 g for the intertidal mussels.

Excretion rate

Excretion rates recorded for the controls in most of the current experiments were high compared to previous work on *P. canaliculus* (Table 8.3). However, the current average excretion of 57.8 µg NH_4^+ g^{-1} hr^{-1} for the intertidal mussels (Table 8.3) compares well to the winter excretion rate of 59.7 µg NH_4^+ g^{-1} hr^{-1} recorded for mid shore intertidal *P. canaliculus* collected from various locations on Banks Peninsula (Marsden and Weatherhead, 1999). Conversely, the current rates were higher than the rates of 15.4 to 38.2 µg NH_4^+ g^{-1} hr^{-1} recorded for a 55 cm length mussels from the Pelorus Sound (James *et al.*, 2001). Compared to the excretion recorded for *Mytilus edulis* and *Mytilus californianus*, the rates recorded in the current study were high (Table 8.3). This may have been related to the high clearance rates seen.

The excretion rate of *P. canaliculus* has been linked to chlorophyll levels in natural ecosystems (James *et al.*, 2001). In the current study, the higher excretion displayed by the intertidal mussels could also have been related to the high clearance rates of these

specimens. In the present study, the excretion rates recorded for intertidal mussels were generally higher than for the farm mussels but the large standard errors suggest that the differences were not based on physiological differences between two types of mussels. Also, previous studies have shown a tendency for excretion in large mussels to decline over time (Bayne *et al.*, 1976b) but this was never seen in the current experiments. This was probably because the mussels in the current experiment were fed.

Table 8.3: Excretion rates determined for *P. canaliculus* and other mussel species.

Species	T (^{o}C)	Excretion rate (µg NH_4^+ g^{-1} h^{-1})	Reference
P. canaliculus	15	15-60	(Weatherhead, 1993)
P. canaliculus	15	15.4-38.2	(James *et al.*, 2001)
Mytilus californianus	13	23.88	(Bayne *et al.*, 1976b)
Mytilus edulis	12	10.7	(Tedengren *et al.*, 1990)
Mytilus edulis (Nov '79-Jan '81)	0-15	2.2-12	(Thompson, 1984)
Mytilus edulis	9	19	(Widdows and Johnson, 1988)
M. edulis (Loch Etive, May 1992)	12.5-13.5	9.5	(Okumus and Stirling, 1994)
M. edulis (Loch Leven, May 1992)	12.5-13.5	10.7	(Okumus and Stirling, 1994)
Choromytilus chorus	12	16.22	(Navarro, 1988)
Mytilus edulis	11-20	5.6	(Martin *et al.*, 1984)
P. canaliculus	15	57.8	Intertidal mussels: Chapters 4-7
P. canaliculus	15	30.2	Farm mussels: Chapters 4-7

SfG

The SfG of mussels can be affected by various environmental and intrinsic factors. For example, SfG of two populations of cultured *M. edulis* was considerably different between sites within 30 km of each other (Okumus and Stirling, 1994). The variability in SfG in a number of studies was believed to be related to environmental factors such as food availability (Okumus and Stirling, 1994; Widdows *et al.*, 1997; Helson and Gardner, 2007) although a genetic basis for physiological differences was also suspected to be important (Tedengren *et al.*, 1990). In the present study, SfG calculated for intertidal *P. canaliculus* was low compared to the 45.6 J g^{-1} h^{-1} (Table 8.4) recorded for specimens collected from

Seatoun in Wellington Harbour (Helson and Gardner, 2007). The high SfG in the previous study may be because losses due to excretion were not determined. Also, the energetic value of 23 J mg^{-1} assigned to the particulate organic matter (POM) was higher than the energetic value of 9.8 J L^{-1} for algal food which was used to calculate the energy intake in the current experiments (Helson and Gardner, 2001).

Table 8.4: SfG for *P. canaliculus* and other mussel species in full salinity seawater.

Species	T (°C)	SfG (J g^{-1} h^{-1})	Reference
P. canaliculus	12.5-18.3	45.6	(Helson and Gardner, 2007)
P. canaliculus	10.4-15.9	-1.3-24.9	(Gardner, 2000)
Mytilus californianus (starved)	13	-3.31	(Bayne *et al.*, 1976b)
Mytilus californianus (fed)	13	12.62	(Bayne *et al.*, 1976b)
Mytilus edulis	11-20	43.8	(Martin *et al.*, 1984)
Mytilus edulis	12	72.6	(Tedengren *et al.*, 1990)
Mytilus edulis (Nov '79-Jan '81)	0-15	2.75-21.83	(Thompson, 1984)
Mytilus edulis	9	2.91	(Widdows and Johnson, 1988)
M. edulis (Loch Etive, May 1992)	12.5-13.5	62.3	(Okumus and Stirling, 1994)
M. edulis (Loch Leven, May 1992)	12.5-13.5	27.5	(Okumus and Stirling, 1994)
Choromytilus chorus	12	20.07	(Navarro, 1988)
M. galloprovincialis	12.5-18.3	1.26	(Helson and Gardner, 2007)
A. maoriana	12.5-18.3	19.1	(Helson and Gardner, 2007)
M. galloprovincialis	10.4-15.9	-4.9- -2.7	(Gardner, 2000)
A. maoriana	10.4-15.9	-4.7-38.2	(Gardner, 2000)
P. canaliculus	15	13.7	Intertidal mussels: Chapters 4-7
P. canaliculus	15	7.4	Farm mussels: Chapters 4-7

Although the SfG values calculated in the current work were lower than for mussels within Wellington Harbor, they were within range of SfG (Table 8.4) of mussels from Island Bay outside of Wellington Harbor (Gardner, 2000). In the present study, the SfG for the farm mussels full salinity controls was 10 J g^{-1} h^{-1} in the cadmium-salinity experiment and 4.9 J g^{-1} h^{-1} in the copper-salinity experiment. On the other hand, the SfG for intertidal full salinity controls was 14.2 J g^{-1} h^{-1} in the cadmium-salinity experiment and 13.2 J g^{-1} h^{-1} in

the copper-salinity experiment. The low averages for the farm compared to the values for the intertidal mussels are the opposite of results which showed that SfG for raft grown *M. galloprovincialis* was 22.71 J g^{-1} h^{-1} versus 13.39 J g^{-1} h^{-1} for intertidal specimens (Labarta *et al.*, 1997).

Differences in SfG may be because of differences in the physiology of intertidal and farm mussels. For example, *Mytilus edulis* collected from littoral sites of the Tamar estuary showed higher SfG than mussels collected from sub-littoral sites from the Swansea docks (Widdows *et al.*, 1984). In another study which compared energy intake of *M. edulis* collected from buoy chains in the Kalunborg Fjord and intertidal rocks in Skovshoved harbour, it was found that the total amount of calories ingested over 24 days was 469 calories for subtidal *M. edulis* versus 1814 calories for intertidal specimens when adequate food was provided (Riisgård and Randløv, 1981). A similar result was found when *Geukensia demissa* which were emersed for 9 hours on a daily basis showed higher clearance rates, absorption efficiencies and ^{14}C ingestion rates than specimens which were not subject to daily emersion periods (Charles and Newell, 1997). This suggested that mussels in the intertidal zone compensated for reduced feeding time by increasing their ingestion rates (Charles and Newell, 1997). In another comparison between intertidal and subtidal *M. edulis*, intertidal mussels were not shown to compensate for reduced feeding time but these specimens had SfG of 32.4 J g^{-1} h^{-1} compared to 18 J g^{-1} h^{-1} for subtidal specimens subjected to intermittent feeding (Widdows and Shick, 1985). A similar situation of higher SfG for intertidal specimens compared to subtidal specimens was described for *P. canaliculus* (Weatherhead, 1993). Therefore, the higher SfG of intertidal mussels seen in this thesis could reflect a greater capacity in the intertidal mussels to maintain higher energy acquisition in dynamic and stressful environments (Bayne, 1998).

Condition index

The condition index has been used to evaluate the nutrition, reproductive status and environmental conditions which bivalves occupy (Crosby and Gale, 1990; Marsden, 2004). Condition of mussels has been expressed as the ratio of the dry tissue weight to the dry shell weight but a standard method using the difference between the fresh weight of the live animal and the dry weight of the bivalve shell has been proposed (Crosby and Gale,

1990). Most of the studies which have used the proposed standard gravimetric method of condition index have been studies on *P. canaliculus* in New Zealand (Table 8.5).

Table 8.5: Condition index determined for *P. canaliculus* and other mussel species.

Species	Temperature °C	Condition index	Reference
P. canaliculus	14.5	100-150	(Lachowicz, 2005)
P. canaliculus	12.5-18.3	142.5	(Helson and Gardner, 2001)
P. canaliculus	12.5-18.3	128.5	(Helson and Gardner, 2001)
Mytilus edulis		143.70-168.70	(Brake *et al.*, 2004)
P. canaliculus	11.4-19.1	350-450	(Hickman *et al.*, 1991)
P. canaliculus	15	74-87	(Weatherhead, 1993)
P. canaliculus	12.5-18.3	100-200	(Helson *et al.*, 2007)
A. maoriana	14.5	100-511	(Lachowicz, 2005)
M. galloprovincialis	14.5	56-152	(Lachowicz, 2005)
P. canaliculus	15	87 ± 4.9	Intertidal mussels: Chapters 4-7
P. canaliculus	15	105 ± 5.0	Farm mussels: Chapters 4-7

On the other hand, few studies have used the suggested standard method to determine condition indices in Europe or North America. As a result, there are few opportunities for comparing the condition determined in this research with condition of other mussel species. However, in one instance when the same method was used, condition of *Mytilus edulis* cultured for 9 and 23 months was found to be higher than condition of both the farm and intertidal mussels in the present study (Brake *et al.*, 2004).

The current study also revealed that the condition index of *P. canaliculus* sourced from a farm in Pigeon Bay were higher than condition of intertidal mussels collected from Taylors Mistake (Weatherhead, 1993) (Table 8.5). The condition determined for the intertidal mussels in the present study were also lower than values recorded in *P. canaliculus* in Wellington and Marlborough (Hickman *et al.*, 1991; Helson and Gardner, 2001; Lachowicz, 2005). The low condition in the present study may be related to the fact that

the condition of *P. canaliculus* can be drastically affected by low availability of food (Hickman *et al.*, 1991). In addition to the difference between intertidal and farm mussels, the condition of intertidal mussels was higher in the summer months compared to the winter months (Table 8.6).

Table 8.6: Condition of farm and intertidal mussels at various months in 2007

Month	Condition Index	
	Intertidal	Farm
January	101.1 ± 5.4	
February	87.3 ± 5.5	
March		144.6 ± 6.5
July	68.3 ± 3.3	
August		79.9 ± 4.4
October	73.3 ± 4.1	
November		91.4 ± 4.3
December	83.8 ± 5.7	

This may be related to build up of the gonads during the summer-autumn months (Buchanan, 2001) when more food is available (Hickman *et al.*, 1991).

8.3 Energetics of the metal and salinity stressed mussels

The primary goal of this research was to answer the question of whether copper or cadmium affected SfG of farm and intertidal *P. canaliculus* differently at high versus low salinity. Copper is believed to be more toxic to marine invertebrates than cadmium (Gorski, 1993) because toxicity of the former is related toxic effects of Cu^{+2} as well as $CuOH^{+}$ while only Cd^{+2} is said to be toxic in seawater conditions (Nell, 2002). Reduced salinity is known to cause significant effects on physiological indices of mussels (Navarro, 1988) but the combination of low salinity and trace metal stresses has not been extensively studied for mussel species such as *P. canaliculus*.

Metal stress

The experiments showed differences in physiological indices caused by sub-lethal concentrations copper and cadmium. The difference in concentrations of these metals which caused a decline in SfG suggests that SfG may be more sensitive to copper. However, there was considerable variation in the physiological rates over time. The principal effect of time was usually between the rates recorded on days three and seven. This effect of time at the start of the experiments was also seen in the physiological rates of the first two intervals for *Perna viridis* exposed to cadmium and zinc (Cheung and Cheung, 1995).

Copper at a concentration of 100 μg Cu L^{-1} was found to clearly affect the clearance, respiration, excretion and SfG of intertidal mussels. Copper only affected the clearance and SfG of farm mussels. These results differ from results which show that the intertidal *Saccostrea cucullata* and *Crassostrea lugubris* oysters were more tolerant to 20 μg Cu L^{-1} than subtidal *Crassostrea belcheri* (Elfwing and Tedengren, 2002a). The current results may have arisen because the basal clearance rate for the intertidal mussels was considerably higher than the clearance for the subtidal farm mussels. Such a difference would have caused greater exposure of the mantle tissue of intertidal mussels to copper. Nevertheless, the effects of copper on both the intertidal and farm mussels can be considered acute sublethal because SfG of both types of mussels was reduced to values less than 20% of the controls.

Cadmium had no effects on clearance, respiration, excretion, SfG or condition of farm or intertidal mussels at concentrations between 33 μg Cd L^{-1}, 66 μg Cd L^{-1} and 99 μg Cd L^{-1}. This has previously been shown for *Mytilus edulis* exposed to 100 μg Cd L^{-1} (Poulsen *et al.*, 1982). However, beginning at concentrations between 500 μg Cd L^{-1} and 1,000 μg Cd L^{-1}, clearance and SfG of intertidal mussels were affected. At a concentration of 1,500 Cd L^{-1}, the clearance and SfG of intertidal mussels and the clearance, respiration and SfG of farm mussels were affected. SfG of intertidal mussels exposed to this concentration of cadmium was decreased to less than 10% of the controls while SfG of farm mussels exposed to this concentration of cadmium was depressed into negative values.

Salinity stress

The mytilid mussels are considered to be euryhaline species capable of surviving in salinities ranging from 4 ppt to 38 ppt (Bayne *et al.*, 1976a). However, when the soft tissues of these mussels were exposed to low salinity, no osmoregulation occurred and the weight of the tissues increase exponentially (Bayne *et al.*, 1976a). Mytilid mussels are therefore considered to be osmoconformers. The ability of mussels to survive in low salinity environments while possessing no physiological capacity for osmoregulation has been attributed to a capacity for tight closure of the valves at salinities below 20 ppt (Davenport, 1981). However, valve closure does not occur without costs. The cost shown in the current experiments was that both intertidal and farm mussels lost their capacity to feed at 17 ppt. Low salinity also resulted in a reduction in respiration rate for farm mussels (Tables 6.2, 7.7). A slight tendency of increased excretion in low salinity was also shown for farm mussels. Combined, these physiological costs resulted in reduced SfG which was proportionally more acute for the farm mussels compared to intertidal mussels. However, beyond having to absorb the costs of reduced feeding, the intertidal mussels displayed a capacity to increase SfG over time when exposed to low salinity.

Combined metal and salinity effects

A number of environmental factors have been shown to affect the degree to which metal contaminants affect invertebrates. For example, uptake of copper by the oyster *Crassostrea virginica* was significantly greater in the presence of *Thalassiosira pseudonana* (Zamuda, 1989). Other important factors that can determine the effects of contaminants include temperature, dissolved organic matter, bicarbonate alkalinity and salinity (USEPA, 2003). Effects of factors such as salinity have not been fully studied but there are indications that the physiological effects of metals can be different at high versus low salinities (Blanchard and Grosell, 2006). For example, cadmium was more toxic at low salinities (Sunda *et al.*, 1978; Fischer, 1988) because the concentration of free cadmium in solution is inversely related to salinity (Engel and Fowler, 1988). As a result, free cadmium represented 20%, 8% and 4.5% of the total cadmium at salinities of 5, 15 and 25 ppt (Hall *et al.*, 1995). In full strength seawater, the percentage of free cadmium (Cd^{+2}) was between 0.65%-3% of the total cadmium (Neff, 2002; De Wolf *et al.*, 2004; Rainbow and Black, 2005). On the other hand, both copper hydroxide ($CuOH^{+}$) and free

copper (Cu^{+2}) are highly toxic in seawater (Neff, 2002). Full strength seawater was found to contain between 0.6% and 5% of the total copper as Cu^{+2} and 0.3% - 7.7 % as $CuOH^{+}$ (Neff, 2002). As a result, copper has been found to be more toxic than cadmium in seawater. In addition, an inverse relationship between bioavailability of these metals and salinity has been established but few studies investigated the physiological implications of this relationship (McLusky *et al.*, 1986).

Some of the physiological implications were investigated in my experiments and showed that when copper and low salinity were studied simultaneously, the interaction between salinity and copper were significant for excretion and SfG of farm mussels but only for clearance and SfG in intertidal mussels. On the other hand, when cadmium and salinity were studied together, the cadmium salinity interaction was significant for clearance, excretion and SfG of intertidal mussels and the clearance, excretion, respiration and SfG of farm mussels. This tendency to detect more interactive effects between cadmium and salinity compared to copper and salinity may be related to different modes of action of cadmium and copper. Copper primarily affects the gills (Redpath and Davenport, 1998) while cadmium causes kidney and metabolic failure and it inhibits uptake of calcium and iron through membranes (Wright, 1995; Schafer *et al.*, 1999).

In the current experiments, the significant interactions between metal and salinity arose because the physiological rates for the metal exposed and metal unexposed low salinity treatments were often similar while the rates for metal exposed and unexposed mussels in full salinity were different. This indicates that salinity was the forcing factor behind the decline in SfG. No notable synergies were detected between salinity and the metals. This has been shown previously for *Trochus maculatus*, a tropical gastropod exposed to 20 μg Cu L^{-1} at reduced salinity (Elfwing and Tedengren, 2002b).

8.4 Conclusions

The SfG determined in these experiments was higher for the intertidal mussels compared to farm mussels. This could have been due to the allometric relationships between mass and physiological indices seen previously for intertidal *Choromytilus meridionalis* mussels in South Africa (Griffiths, 1979). However, this could also have been due to greater

tolerance in intertidal mussels to laboratory conditions (Bayne and Thompson, 1970) or more rapid recovery of clearance after manipulation (Widdows and Shick, 1985).

Another important result of these experiments was that intertidal mussels exposed to 17 ppt showed capacity to increase SfG over fourteen days of exposure while farm mussels were not able to increase SfG if exposed to low salinity. This may be related to the presence of thirteen leucine aminopeptidase alleles in *Perna canaliculus* sampled along a salinity gradient in Wellington harbor (Gardner and Kathiravetpillai, 1997) versus seven alleles found in mussels from marine habitats (Sin *et al.*, 1990). Comparisons between intertidal and subtidal *P. canaliculus* also showed that over distances exceeding 100 km, intertidal populations were more heterozygous compared to subtidal specimens (Sin *et al.*, 1990) suggesting that the differences in SfG for farm and intertidal mussels in this study could have been based on intrinsic factors. The greater adaptability of the intertidal specimens documented in this thesis -coupled with the knowledge that these intertidal specimens were more heterozygous- suggests that the *P. canaliculus* conforms to the niche-width variation hypothesis which advocates that populations occupying more stressful conditions will be more genetically rich (Valen, 1965).

The data presented in this thesis also suggest that it would be inappropriate to use SfG to explain the growth rates of farm versus intertidal mussels. Part of this is because no factor is applied for the low tide period when intertidal mussels cannot feed (Bougrier *et al.*, 1998). Secondly, SfG only models for total energy and does not account for limiting nutrients or ration balance which may determine growth efficiencies and the maximum amount of weight gain possible (Grant and Cranford, 1991; Guillaume *et al.*, 2001). This suggests that considerations relating SfG with variables such as protein, or HUFA content of bivalve feeds may be important (Navarro *et al.*, 2000; Guillaume *et al.*, 2001). .

Although the current study was one of the first to compare SfG of farm and intertidal mussels, it is possible that the high SfG for intertidal mussels may have been an overestimations of true growth potential in their habitats (Grant and Cranford, 1991; Weatherhead, 1993). This can be explained by (1) Higher compensatory feeding ability in the intertidal mussels (Charles and Newell, 1997; Bayne, 1998; Marsden and Weatherhead, 1999) and (2) Downward regulation of feeding rates in the farm mussels in conditions of high chlorophyll or total particulate matter (Scholten and Smaal, 1998; Hawkins *et al.*, 1999).

High compensatory ability refers to tendency in some bivalves to show high respiration and clearance rates after a period of nutritive stress (Bayne *et al.*, 1976a). It has also been shown that the clearance of *Choromytilus meridionalis* increased for specimens offered only low rates of ration (Griffiths, 1980b). It is possible that this is a trait linked to intertidal mussels which are unable to feed during low tide (Charles and Newell, 1997). Recent studies on the mussel *Yoldia hyperborea* have reconfirmed a rapid response to food using the siphon position and length as response variables (Stead and Thompson, 2006).

On the other hand, downward regulation of feeding rate is more likely to be a trait associated with fast growing farm mussels having uninterrupted access to particles traversing submerged ropes. Laboratory studies have shown no differences in clearance, absorption efficiency and SfG between raft and wharf *P. canaliculus* brought into laboratory settings (Gardner and Thompson, 2001). In another study, no compensatory action between absorption efficiency and clearance rates were found in mussels fed at various levels of seston (Gardner, 2002). However, when measurements were done on field samples, researchers in the Marlborough region showed that the clearance rate of farm mussels was maximum at a narrow range of 1-2 μg chlorophyll L^{-1}, and declined sharply at higher and lower concentrations of chlorophyll and total particulate matter (Hawkins *et al.*, 1999). This down regulation of clearance was thought to operate in tandem with improved particle selection. This feedback loop allowed farm mussels to prevent overloading of the filtration mechanism and maintain high growth rates (Hawkins *et al.*, 1999). A similar example included reduced clearance for *Mytilus edulis* occupying waters with high total particulate matter (Prins *et al.*, 1991). It is possible that the intertidal *P. canaliculus* used in the current research may not possess the capacity to regulated feeding so tightly and are probably less efficient growers as a result (Hickman, 1979).

My experiments also showed that SfG is not equally affected by copper and cadmium. In the case of copper, clearance, respiration, excretion, and SfG of *P. canaliculus* were significantly affected by 100 μg Cu L^{-1}. On the other hand, these indices, including the SfG, were not affected by concentrations of cadmium below 500 μg Cd L^{-1}. At 1,500 μg Cd L^{-1}, clearance, respiration and SfG of farm mussels were affected but only clearance and SfG of intertidal mussels were affected. These results confirm that copper is a more potent toxin than cadmium.

My experiments also showed that when farm or intertidal mussels were exposed to acute concentrations of copper or cadmium, the decline in SfG was equally severe for farm and intertidal mussels, although the decline due to copper was delayed in farm mussels in good condition. This indicates that, unlike the case with salinity, the separate origin of the mussels did not confer greater tolerance to acute concentrations of either copper or cadmium with time.

These results of the present study are important because there are indications that trace metal concentrations are increasing in New Zealand (Long *et al.*, 1995; Stevens and Forrest, 1996; Winchester, 1998; Perera, 2004; Harding, 2005; Hauraki Gulf Forum, 2008). In a number of cases, when such increases of highly toxic trace metals have not been monitored, ecosystem-wide effects including local species extinctions have been noted for bottom dwelling invertebrates (Alve, 1991) as well as intertidal mussels (Bryan *et al.*, 1986; Cadee *et al.*, 1995) near large cities. As a result, programmes such as the International Mussel Watch were developed to compile data on the background levels of important toxins at most large cities in the world (Goldberg and Bertine, 2000; Tibbetts, 2002; O'Connor and Lauenstein, 2005). These bioaccumulation testing programmes are costly to maintain and they do not take into account the interactions which occur among biotic, abiotic and anthropogenic factors which may affect recruitment and survival of species competing for similar resources. Also, bioaccumulation studies cannot detect behavioural changes which can result from natural and anthropogenic stressors. Therefore, methods which integrate factors such as duration of exposure, salinity and concentration of trace metals such as cadmium need to be developed to ensure more comprehensive and cost effective environmental assessments (Sarà *et al.*, 2008).

The results from the current experiments suggest that intertidal *P. canaliculus* could be successfully used as a sentinel at coastal locations where copper pollution is known to be a risk but where the concentrations at any point in time would be low. In such situations, naturally occurring intertidal *P. canaliculus* could be deployed in cages to control and monitoring sites near to marinas, dry-docks or industrial ports and used to detect environmental changes. The deployed mussels would be periodically sampled and the temporal trends in SfG compared to the SfG of the control populations located at pristine sites. This could be a new use for New Zealand's endemic *P. canaliculus*. In the future, such studies could investigate physiological effects of effluent from the Christchurch's

new ocean outfall using the techniques in this thesis. Intertidal and farm mussels could be transplanted to buoys chains on the outfall and the clearance, respiration, excretion and SfG determined after fixed periods of exposure. Also, it may also be possible to use the methods to assay for newly suspected aquatic pollutants (Lau *et al.*, 2007) or investigate physiological effects of new "clean/green" antifouling products (Forrest *et al.*, 2007) which could be developed by New Zealand's chemical industry.

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